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Difluorinated analogues of oxaloacetate and aspartate as enzyme probes.

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DIFLUORINATED ANALOGUES OF OXALOACETATE AND ASPARTATE AS ENZYME PROBES.

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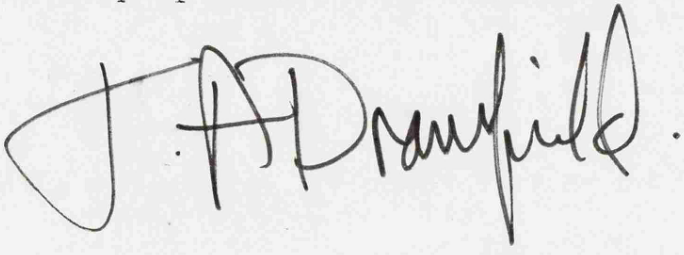

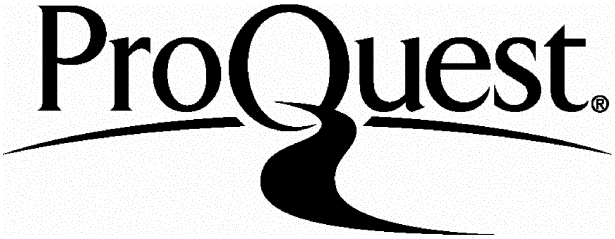

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SUMMARY

L-Difluoro-aspartic acid was prepared by the enzymic transamination of difluoro-oxaloacetate using aspartate aminotransferase (AAT). The purification and some physical properties of this novel compound are reported. The chemical synthesis of D, L-difluoro-aspartic acid was attempted.

The interaction of difluoro-aspartate with pig heart cytosolic AAT was studied. The observed potent inhibition by this compound in steady state kinetic experiments was interpreted as being due to the formation of an aldimine-AAT-difluoro-aspartate complex, an aminic-AAT-difluoro-aspartate complex and an enzyme-substrate-difluoro-aspartate ternary complex. The apparently aberrant formation of the latter two complexes is discussed.

The interaction of difluoro-aspartate with the aldimine form of AAT was studied in detail. A complex of difluoro-aspartate with aldimine-AAT was detected and its dissociation constant determined by spectral titration. The spectrum of the complex was shown to be distinct from that of aldimine enzyme with an absorption maximum at 340nm the magnitude of which was insensitive to pH, and also a minor absorption band at ca 430nm. The pH variation of the dissociation constant was analysed. Stopped flow experiments provided lower bounds for the rate of formation and dissociation of the complex.

Preliminary experimentation indicated the possibility of a very slow productive breakdown of the aldimine-AAT-difluoro-aspartate complex to yield an undentified keto-acid. The inefficiency of difluoro-aspartate as a substrate for AAT is discussed.

Tentative conclusions about the molecular nature of the complex were drawn from the results of a circular dichroism study of the binding of difluoro-aspartate to aldimine-AAT and from an attempt to detect a difluoro-aspartate aldimine Schiff base by borohydride reduction. A molecular reaction scheme involving the tetrahedral transaldimination intermediate in equilibrium with a small amount of the difluoro-aspartate aldimine Schiff base is proposed.

The pH variation of the kinetic parameters for the slow transamination reaction of difluoro-oxaloacetate with aminic-AAT was studied as a model for the pH variation of the transamination reaction. The observed invariance of Km and Vm with pH for the above reaction is discussed.

CONTENTS

PART 2 DISCUSSION

Introduction : Aspartate Aminotransferase

Transamination may be defined as the intermolecular transfer of an amino group from an α-amino acid to an α-keto acid, without the intermediate formation of ammonia. Enzymic transamination between glutamic and pyruvic acids in pigeon flight muscle was first demonstrated by Braunstein and Kritzman in 1937. Subsequent studies, demonstrating that most naturally occurring amino acids undergo such reactions and outlining their importance in amino acid metabolism, have been reviewed (Braunstein, 1947; Cohen, 1951; Meister, 1955; 1957).

One enzyme responsible for a transamination reaction is aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, E.C.,2.6.1.1.). Aspartate aminotransferase (AAT) catalyses preferentially the reversible interconversion of the substrate pairs L-glutamate/2-oxoglutarate and L-aspartate/oxaloacetate. This enzyme is particularly active in heart tissue and pig heart ventricles are a convenient source material.

AAT exists as two genetically distinct isoenzymes, cytoplasmic (anionic) and mitochondrial (cationic), (Wada and Morino, 1964). These isoenzymes differ immunologically, chemically, physically, and in their dynamic properties (Wada and Morino, 1964; Martinez-Carrion and Tiemeier, 1967; Wada et al., 1968; Michuda and Martinez-Carrion, 1969; 1970; Martinez-Carrion et al., 1970a). The cytoplasmic enzyme has been most commonly prepared (Banks et al., 1961; 1968a; Jenkins et al., 1959a; Lis, 1958; Jenkins, 1962; Wada and Morino, 1964), and that of pig heart is the most studied of

enzymes catalysing transamination reactions.

An important contribution to the understanding of the mechanism of enzymic transamination came from the studies of chemical transamination. Prior to the discovery of enzymic transamination a chemical transamination between α-aminophenyl-acetic acid and pyruvic acid was observed (Herbst and Engel, 1934). This reaction differs from enzymic transamination in that it requires extreme conditions and is not reversible because decarboxylation of the amino acid occurs. Esterification of carboxyl groups however can prevent decarboxylation (Brewer and Herbst, 1941). With major modifications chemical transamination reactions were ultimately to be utilized as congruent chemical models of enzymic transamination.

Snell (1944; 1945) demonstrated a reversible transamination reaction between glutamate and pyridoxal yielding pyridoxamine and 2-oxoglutarate. This reaction occurring at physiological pH led Snell to suggest that pyridoxal and pyridoxamine might be involved in enzymic transamination. The first direct evidence to suggest the involvement of these compounds came from observations that transaminase activity was diminished in Vitamin B6 deficiency (Schlenk and Snell, 1945). Other evidence for this supposition followed (Green et al., 1945; Lichstein et al., 1945; Kritzman and Samarina, 1946; Schlenk and Fisher, 1947; Meister et al., 1953), indicating the requirement of pyridoxal 5'-phosphate (PALP) for enzymic transamination. Highly purified AAT was ultimately shown to contain bound PALP which is essential for enzymic activity (Jenkins et al.,

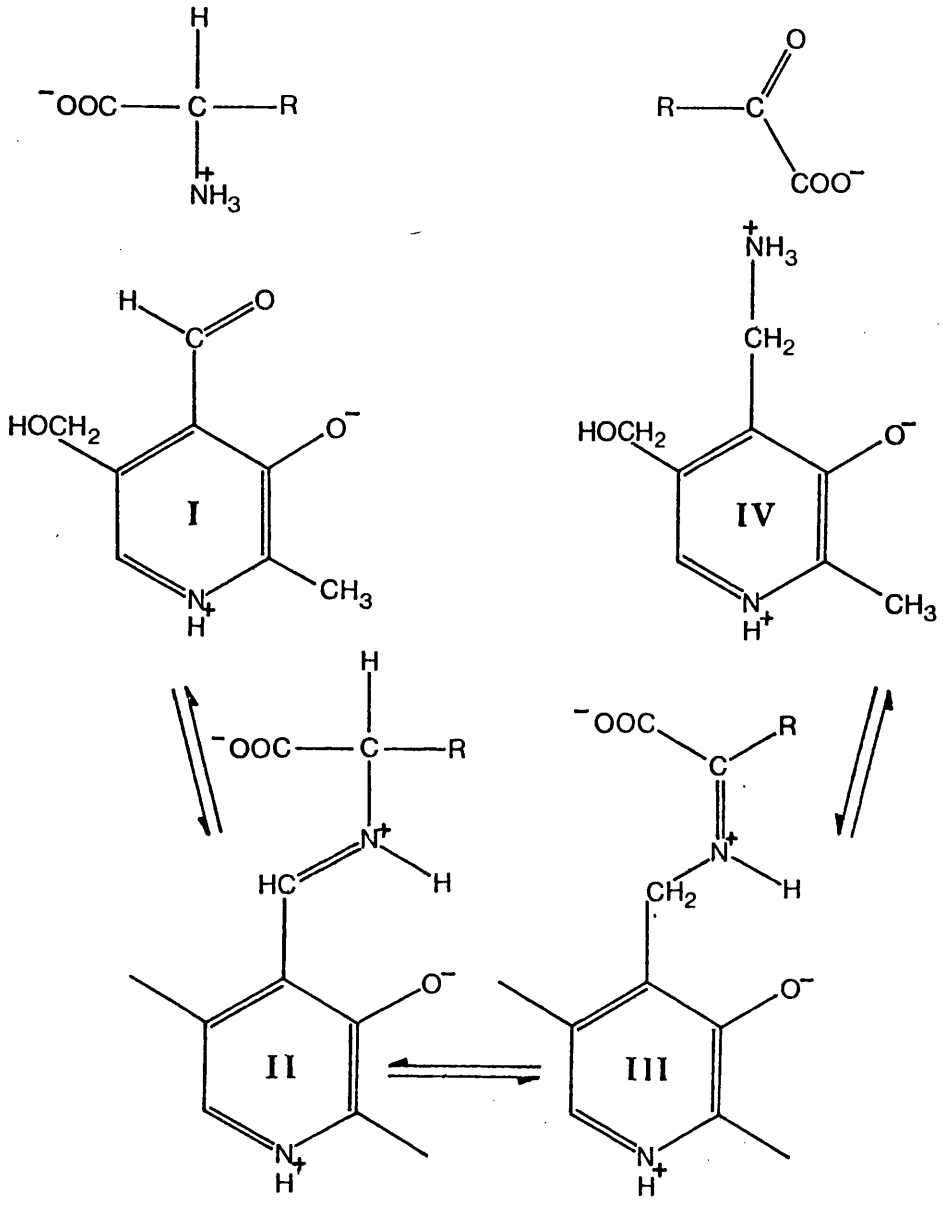


Figure 1

Mechanism of pyridoxal linked transamination according to Metzler et al., (1954).

1959a). Thus the coenzymic role of PALP provided the impetus for chemical investigation of the reaction of pyridoxal with amino acids in order to explain the detailed mechanism of enzymic transamination. Such studies led to the formulation of a general mechanism for pyridoxal-linked amino acid transformations (Braunstein and Shemyakin, 1953; Metzler et al., 1954; Braunstein, 1960).

The proposed mechanism is outlined in Figure 1. It involves condensation of the amino acid with pyridoxal (Fig. 1, I) to form an aldimine Schiff base (II). This results in electron withdrawal from the amino acid α-carbon into the electron sink of the protonated pyridine ring with a consequent weakening of the Cα-substituent bonds. This can lead to a number of reactions, one of which is transamination. Transamination is the result of proton transfer from the α-carbon to the coenzyme 4'-carbon yielding a ketimine (III). The ketimine can be hydrolysed to yield the keto acid and pyridoxamine (IV).

Further studies with model systems led to the refinement of the understanding of the basic chemical feature of transamination. There are many indications that enzymic transamination operates by the same general mechanism ((refer Fig. 1), Jenkins and Sizer, 1957; Snell and Jenkins, 1959; Lis et al., 1960; Hammes and Fasella, 1962; Banks et al., 1963b; Braunstein, 1964; Guirard and Snell, 1964), further details of which are discussed in this thesis.

AAT contains two molecules of bound coenzyme (PALP) per molecule of protein (Jenkins et al., 1958; Lis et al., 1960), the enzyme being a dimer of two identical sub-units (Martinez-Carrion and Tiemeier, 1967; Polyanovsky, 1965;

1968; Ovchinnikov et al., 1973), which are not linked by covalent bonds and may be dissociated (Polyanovsky, 1965; 1968; Polyanovsky et al., 1964; 1965). All available evidence points to a highly ordered, compact conformation of the molecule (Braunstein, 1973). Under certain conditions the sub-units may act asymmetrically (Christen and Riordan, 1970; Arrio-Dupont, 1972; Cournil and Arrio-Dupont, 1973; Bayley and Harris, 1975; Harris and Bayley, 1975): but there is no evidence for site-site interactions (Braunstein, 1973), and work with half-active hybrid enzyme dimers essentially dispels this possibility, (Schelegel and Christen, 1974; Boettcher and Martinez-Carrion, 1975; 1976; Boettcher, 1976).

The molecular weight of AAT was for some time in contention but a value of 93,147 based on amino acid sequence is now available (Ovchinnikov et al., 1973).

Both isoenzymes of AAT exist as subforms (Martinez-Carrion et al., 1965) and those of the cytoplasmic enzyme have been studied in some detail by Martinez-Carrion et al., (1965, 1967a and 1970a). These authors have demonstrated that the enzyme is separable by starch gel electrophoresis into four fractions containing AAT activity, designated α, β, γ and δ in order of their increasing anodic mobilities. The subforms isolated by CM Sephadex chromatography do not differ appreciably in their molecular size, primary structure or immunoprecipitation behaviour, but do differ in their specific activities and absorption spectra in the visible region. Specific catalytic activity of the subforms is in the order $\alpha > \beta > \gamma$, and is inversely related to the amount of coenzyme bound in an inactive form which is char-

acterised by an absorption spectrum with λ_{max} 340 nm. Approximately half of the coenzyme in the β-subform (Evans and Holbrook, 1974) and most in the γ-subform is bound in this manner. α-AAT is subject to ageing and to urea-induced changes in its electrophoretic mobility. These changes are concomittant with increase in absorption at 340 nm and loss of enzyme activity and may be prevented by thiol-protecting reagents (Arrio-Dupont et al., 1970). Thus the theory has been advanced that the subforms are different conformers arising from an original, active α-form (Banks et al., 1968a; Arrio-Dupont et al., 1970) but the observations of Denisova and Polyanovskii (1974) are contrary to this idea. They have characterised the subforms in terms of differing amounts of neutral and ionogenic sugars. The α-subform possessing least carbohydrate cannot therefore yield the other subforms by conformational changes as proposed.

The subforms are found to exist in cold water homogenates taken from a single heart within 90 minutes of death (Martinez-Carrion et al., 1967a), and so apparently exist in the living cell. The reason for their existence remains unclear however.

Homogeneous preparations of the α-subform of AAT are utilized for the precise measurements of the kinetic and optical parameters of this enzyme.

Transamination involves the conversion of enzyme-bound pyridoxal 5'-phosphate (PALP) into pyridoxamine 5'-phosphate (PAMP) by an amino acid. Net transamination results from the reverse reaction involving the keto acid of the other substrate pair. This the shuttle mechanism

for transamination, was suggested by Schlenk and Fisher (1947) and validated by Jenkins and Sizer (1957; 1960).

AAT containing either PALP or PAMP as the coenzyme (Jenkins and Sizer, 1960) can be isolated. With PAMP as the prosthetic group AAT exhibits a pH-independent absorption maximum at 332 nm whereas the PALP form of the enzyme is colourless at pH values greater than 7 (λ_{max} 360 nm) and bright yellow (λ_{max} 430 nm) below this pH value. This transition is caused by the addition of a single proton to the chromophore, a change having pK_a 6.3 as reported by Sizer and Jenkins (1963). Visible absorption bands of the enzyme-bound coenzyme are optically active (Torchinsky and Korrenva, 1963; Fasella and Hammes, 1964) and because PALP itself is not inherently optically active this phenomenon must arise from the asymmetric interaction of the coenzyme with its apoenzyme. Optical activity of the chromophore will be examined in more detail in the Discussion section.

PALP-AAT absorption characteristics, though different from those of free PALP, resemble those of PALP imines (Metzler, 1957). Thus it was suggested that the coenzyme is bound to a free amino group on the enzyme in an imine-linkage via its formyl group (Jenkins and Sizer, 1957; Jenkins et al., 1959b). An aldimine linkage to the ε-amino group of a lysyl residue was confirmed by reduction of the imine with sodium borohydride and isolation of ε-N-pyridoxal lysine after total acid hydrolysis (Hughes et al., 1962). The lysine residue has been identified as Lysine-258 (Ovchinnikov et al., 1973). The lack of such a covalent linkage in PAMP-AAT is reflected in the greater ease of

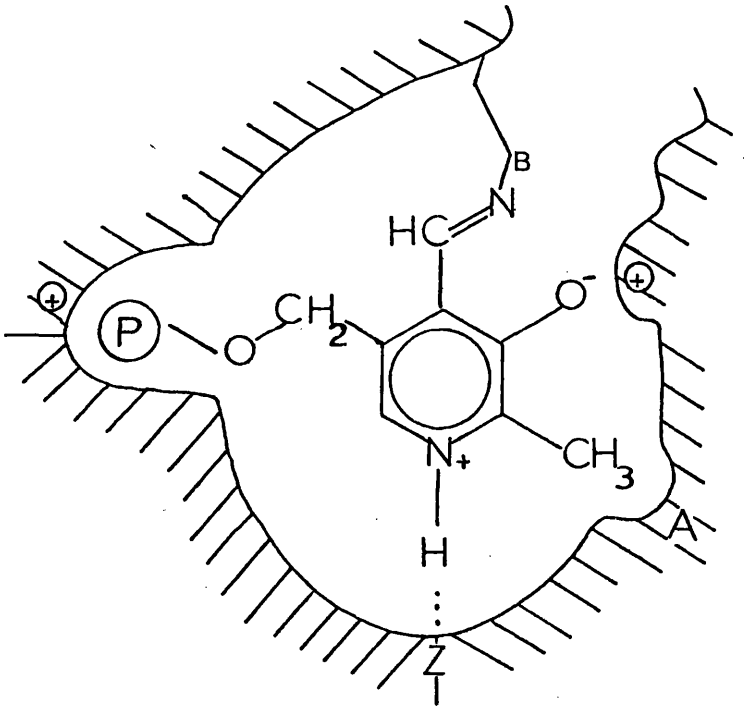
resolution of the coenzyme in this form, a fact which is utilized in methods for preparation of the apoenzyme (Wada and Snell, 1962; Scardi et al., 1963; Dixon and Severin, 1968; Martinez-Carrion et al., 1970a).

The key questions in the study of the mechanism of enzymic transamination relate to how the chemically ill-defined apoenzyme enhances the already existent catalytic activity of the coenzyme. A particular example of such an enhancing factor is given by the lysine-aldimine linkage in that amino acids have been shown to form aldimines more rapidly by transimination with pyridoxal, than by condensation with free pyridoxal in model systems (Jenks and Cordes, 1963).

Other linkages between the coenzyme and protein have been indicated by studies with coenzyme analogues. The phosphate residue of the coenzyme appears to be bound by electrostatic interactions to the apoenzyme as shown by the observation that pyridoxamine has less than one thousandth of the affinity for apo-AAT than does PAMP (Wada and Snell, 1962). When saturated with pyridoxamine, the enzyme also exhibits 1/1,000 of the turnover rate of the intact holoenzyme, demonstrating that the phosphate group is not only required for efficient binding but also for effective catalysis. It would appear that the phosphate group is in the monoionic form, because this form of inorganic phosphate competes with the co-factor for association with the apoenzyme (Banks et al., 1963b).

The nitrogen atom of the pyridine ring has been suggested to be a further binding point. Snell and Jenkins (1959) mooted that the interaction of a proton donating

Figure 2



The binding of PALP to the active site of AAT, after Braustein (1970).

⊕ , cationic protein groups; A, hydrophobic locus ;

B, lysine residue; Z, tyrosine residue.

group (ZH in Fig. 2) with the pyridine ring could partially explain the observed difference in protolytic dissociation between free PALP imines (pK_a 10.5, Metzler (1957) and the enzyme-bound internal aldimine (pK_a 6.3, Sizer and Jenkins (1963)). A positively-charged nitrogen would be expected to reduce the electron density on the Schiff base nitrogen so making it less easily protonated. Circular dichroism studies and studies with the N-oxide derivative of PALP have given supportive evidence for this binding point and implicated a tyrosine residue (Bocharov et al., 1968; Ivanov and Karpeisky, 1969), as the proton-donating group on the enzyme.

As part of a dynamic model for enzymic transamination (to be discussed later) Ivanov and Karpeisky (1969) have considered the plausability of an electrostatic interaction between a cationic group on the enzyme and the anionic phenol group. This, they say, would further help to explain the observed low pK_a of the internal aldimine compared with that of free imines; the protonated pyridine nitrogen accounting only for a lowering of the pK_a from 10.5 to 8.0. The presence of a cationic group in the proximity of the aldimine linkage could lower its pK_a still further to the observed value of 6.3. Studies on the interaction of the 3-desoxy and 3-O-methyl analogues of PALP with apo-AAT support, but do not prove, this hypothesis (Mora et al., 1972). The presence of the 3-OH group is found to be essential for activity in model systems (Snell and Jenkins, 1959).

The involvement of the pyridine 2-methyl group in a hydrophobic interaction has been indicated by kinetic

studies of the interaction of 2-alkyl homologues of PALP with apo-AAT (Bocharov et al., 1968).

Thus the coenzyme appears to be bound by most of its functional groups (Fig.2). According to Ivanov and Karpeisky (1969) tight binding of the coenzyme maintains PALP in the thermodynamically unfavourable highly-reactive bipolar form.

A variety of techniques has been used to study the reaction of AAT with its substrates. For example, the steady state kinetics of the catalytic reaction of AAT have been extensively studied (Velick and Vavra, 1962a, b; Banks et al., 1963b; Wada and Morino, 1964; Henson and Cleland, 1964; Nisselbaum and Bodanskii, 1966; Haarhoff, 1969). All the results quoted are consistent with the shuttle mechanism in which the substrates bind sequentially to form only binary complexes; substituted enzyme, PAMP-AAT, being the intermediary carrier of the amino group. Kinetics of this type are termed "ping-pong, bi bi" (Henson and Cleland, 1962) and have been confirmed, in this case, by product inhibition studies (Henson and Cleland, 1962; Velick and Vavra, 1962b). Included in the work of Velick and Vavra in 1962 are studies of keto-acid inhibition and of the effect of pH on the steady state kinetic parameters. Analysis of the latter by standard procedures suggested that a group on the enzyme with pK_a 6.3 affects the association of enzyme and amino acid substrate. The similarity of the pK_a to that of the 430-360 nm transition (pK_a 6.3, Sizer and Jenkins, 1963) of aldimine enzyme suggested that only the non-protonated enzyme interacts with the amino substrate. The maximum velocity is constant in the range

6-9 and then decreases with a pK_a of 5.1.

Equilibrium spectrophotometric studies of the reaction of AAT with its substrates have provided useful information on the mechanism of transamination. It was by such methods that the first direct evidence for the shuttle mechanism was obtained (Jenkins and Sizer; 1960). These techniques, relying on the unique visible absorption properties of the coenzyme, permit the determination of the binding affinities for productive and non-productive enzyme-substrate complexes and of the equilibrium constants for each half reaction (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966b)

In the presence of substrate, absorption maxima in the regions of 330, 365, 430 and 490 nm have been observed. Absorption maxima at 360 and 430 nm have been attributed to substrate aldimine Schiff base intermediates. Those at 330 and 490 nm have been attributed to substrate ketimine Schiff base intermediates and to a quinoid type intermediate respectively (Fasella et al., 1966).

Saturation of the enzyme with substrates also leads to changes in the optical activity of the chromophore. Absorption bands at 430 and 360 nm are adichroic, those at 330 nm are weakly dichroic and those at 490 nm exhibit negative dichroicity (Torchinsky et al., 1968; Martinez-Carrion et al., 1970a).

The existence of enzyme-substrate Schiff bases has been proved. Reduction of these imines with sodium borohydride, in a manner analogous to the reduction of the internal aldimine, yielded the expected pyridoxyl-glutamate and pyridoxyl-aspartate derivatives on resolution (Riva et al.,

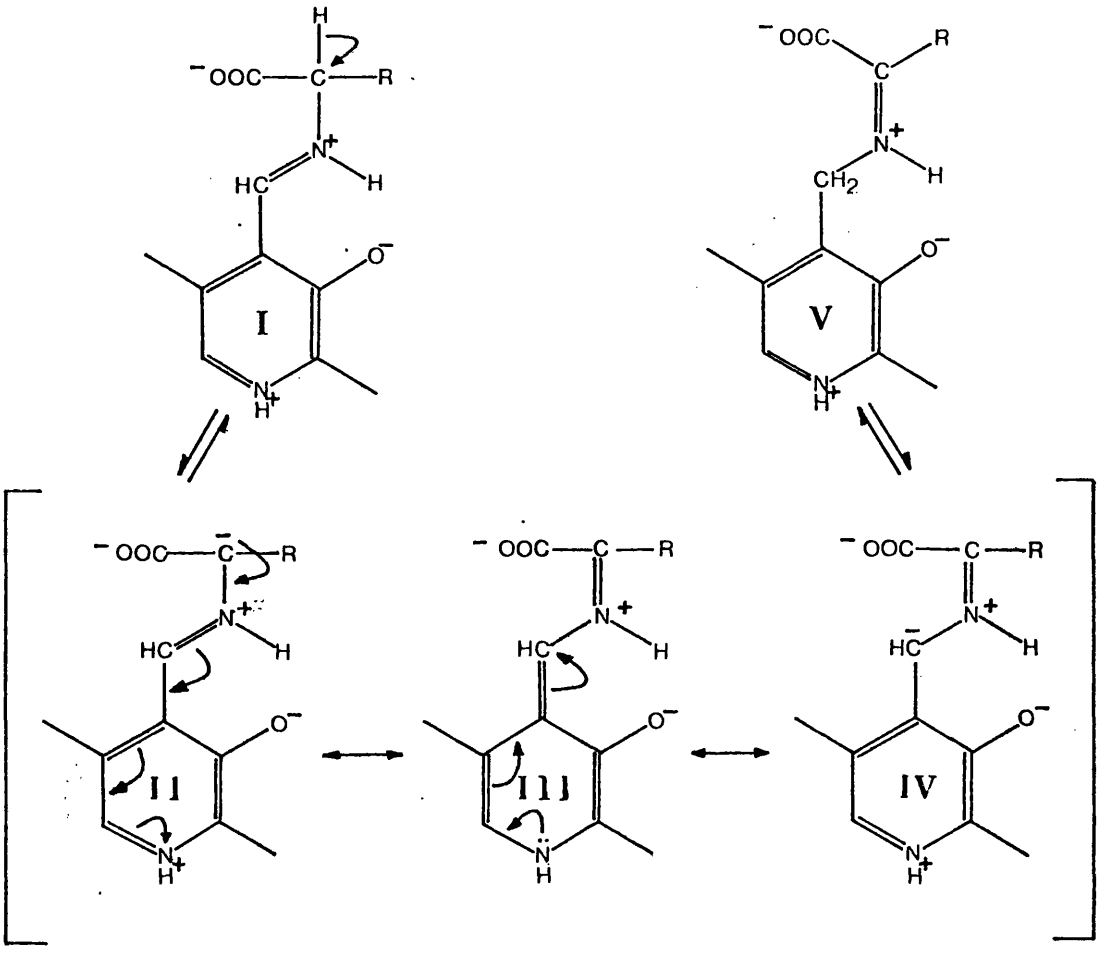


Figure 3

Mechanism for the tautomeric interconversion of substrate aldimine (I) and ketimine (V), after Braunstein (1973).

1964). However experimental details have not been given by Riva et al.

The quinoid-type complex is believed to be an intermediate in the tautomeric interconversion of substrate aldimines (Fig.3, I) and ketimines (V). Removal of the substrate α-carbon proton in the aldimine leaves a carbanion with delocalised negative charge. The resonance states of this carbanion include the α-C carbanion (II), the quinoid intermediates (III) and C-4' carbanion (IV). The latter form may accept a proton to form the ketimine (V).

Evidence that aldimines and ketimines are interconverted by labilization of the substrate α-C-H bond (Metzler et al., 1954; Matsushima and Martell, 1967a) with the intermediate formation of a carbanion (Gram and Guthrie, 1965; Auld and Briuce, 1967) that is resonance-stabilized by a quinoid-type intermediate (Schirch and Slotta, 1966) have been obtained from model systems. However with regard to the enzyme reaction the existence of carbanionic intermediates is in contention. Studies with the redox indicator hexacyanoferrate III indicate an oxidisable carbanion on AAT in the presence of substrates (Healy and Christen, 1973). Increased consumption of tetranitromethane by AAT in the presence of substrates has been similarly interpreted (Shlyaphikov and Karpeisky, 1969) although it has been shown that a syncatalytic modification of tyrosine and cysteine residues with tetranitromethane can occur (Christen and Riorden, 1970; Bocharov et al., 1973; Birchmeier et al., 1973b).

On the other hand, studies on the effect of α-deuterium-substituted amino acids on all six of the

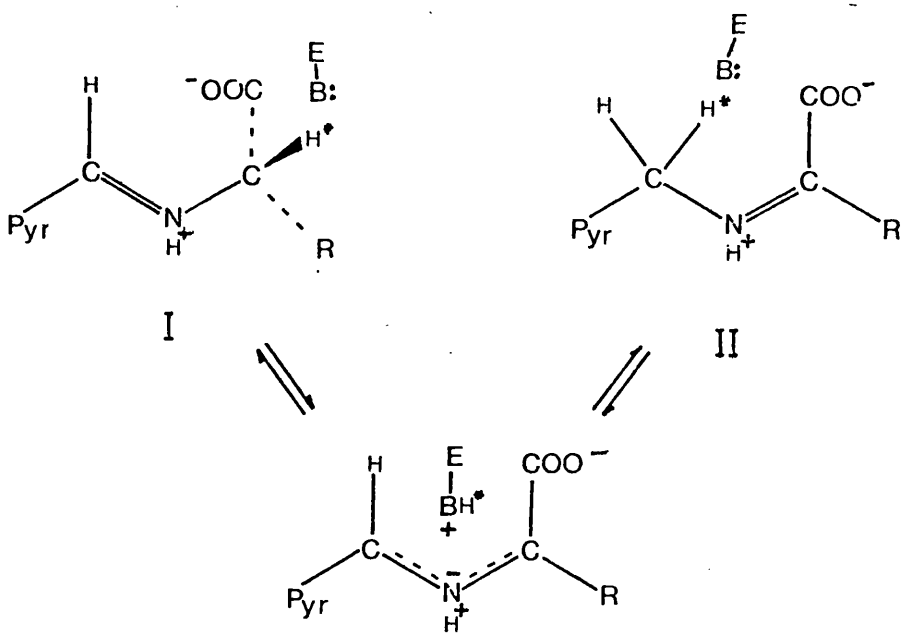


Figure 4

Conformation about the C^{α}-N bond and the stereochemical path of the aldimine $\rightleftharpoons$ ketimine transformation in enzymic transamination, from Dunathan (1971).

Michaelis parameters of AAT have been interpreted to exclude a carbanionic intermediate (Banks et al., 1968b; Doonan et al., 1970). The primary kinetic isotope effect indicated that the rate-limiting step of transamination was the labilization of the α-carbon proton and analysis of this effect provided the four rate constants for the aldimine-ketimine tautomerization step in both half reactions.

The stereochemical course of the reaction has received attention. Dunathan (1966; 1970; 1971) has considered the geometrical variables determining the stereochemistry of Schiff base inter-conversion. . The configuration at C_α is defined by the stereospecificity of AAT for L-amino acids (S symmetry for H). The reaction was shown to be stereospecific for proton removal and addition at C-4', the pro S proton being labile (Dunathan, 1968a, b). According to Dunathan (1966) labilization of the C_α and C-4' protons, in the aldimine and ketimine intermediates respectively, is facilitated when the bond to be broken is perpendicular to the π-electron system of the cofactor. It follows therefore that the possible conformations about C-4'-N in the ketimine and C_α-N in the aldimine are limited to two each, 180° apart.

In bacterial pyridoxamine-pyruvate transaminase, Ayling et al., (1965) provided evidence for the intramolecular cis transfer of the proton by an acid-base group via an intermediate ion pair (Fig.4). Consideration of the percentages of deuterium (in H_2O) and of proton (in D_2O) transfer suggested that the transfer may occur via a lysine residue.

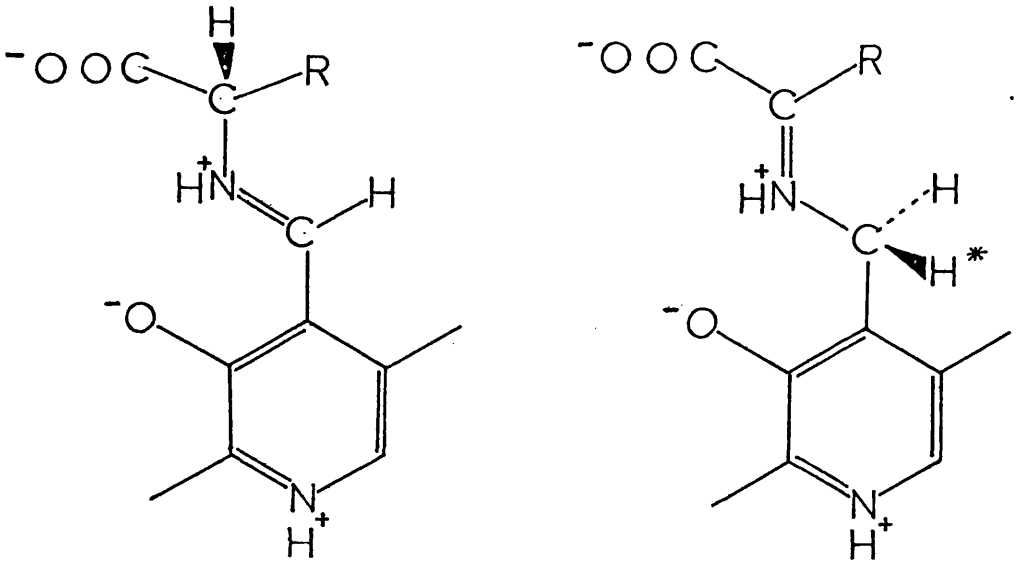


Figure 5

Stereochemistry of the aldimine (1) and ketimine (2) intermediate, according to Dunathan (1971).

No such conservation of the mobile proton was observed with AAT, and cis transfer for this enzyme has only been indicated by indirect means. Substrate aldimine and ketimine Schiff bases are reduced by borotritide with the same stereochemistry as in the enzyme reaction (Besmer et al., 1971). This indicates reduction only on one exposed face of these Schiff bases, and unless this exposed face changes with their interconversion, it also implies a cis mechanism for the tautomerization step. Finally with assumption of a trans conformation about C-4'-N, Dunathan (1970; 1971) was able to define the stereochemistry of the aldimine and ketimine intermediates (Fig.5) and of their interconversion.

The kinetics of the fast intermediary steps in enzymic transamination have been studied by rapid reaction techniques. The reaction of high concentrations of AAT with each substrate has been studied in the stopped flow spectrophotometer (Gutfreund et al., 1961; Hammes and Fasella, 1962). The half time of the reaction was shown to be <5ms. This value, being close to the resolution time of the instrument, permitted only estimation of lower bounds for the bimolecular rate constants.

The technique of temperature jump, with a shorter resolution time, has been somewhat more fruitful. Hammes and Fasella (1962), and later Fasella and Hammes (1967) with improved apparatus and using only the α-subform of the enzyme, have studied the reaction of AAT with its substrates by this method. Three relaxation processes were observed for the reaction of the enzyme with each amino-keto acid pair.

The concentration dependence of the slowest step indicated an intramolecular process and it was suggested that this process must be related to the rate limiting step. The wavelength dependence of the amplitude of the relaxation process suggested that intermediates with spectral maxima 430, 360 and 490 nm were on one side of the rate limiting step and that intermediates with an absorption maximum at 330 nm were on the other side. The concentration dependence of the slowest process was analysed to provide kinetic parameters for the reaction. This analysis, assisted by auxillary data, was based on the assumption of only two intermediates with their interconversion being decisive in controlling the slowest relaxation process. Four rate-controlling first order rate constants were obtained (two for each half reaction). If the number of postulated intermediates was greater than two, as was suggested, these rate constants would only be lower bounds. Four binding constants defining the concentration dependence of the slow relaxation process were also calculated. The two remaining relaxation processes (for each half reaction) were too rapid for quantitative measurement. Nevertheless an analysis was argued which allowed the estimation of lower bounds for the bimolecular association rate constant and for the dissociation rate constants of keto-acid and amino acid with AAT. It was pointed out that the second order rate constant for the association of amino acid with AAT was close to the maximum value possible assuming a diffusion-limited process.

In conclusion Fasella and Hammes (1967) suggested that the most profitable approach for obtaining any further

mechanistic information on AAT was by studies of the interaction of AAT with pseudo substrates. The interaction of pseudo substrates with the enzyme has provided much valuable information on the mechanism of AAT.

Those substrate analogues that have provided information on the formation of enzyme-substrate aldimine complexes will be discussed first. Use of substrate analogues has indicated that all of the functional groups of the natural amino-acid substrates (i.e. α-Carboxyl, ω-Carboxyl and the α-amino group) contribute to the binding with AAT. Hydroxylamine and thiosemicarbazide, which can be considered as substrate amino group analogues, have been shown to bind to the active site, even though they do not possess carboxylate groups (Velick and Vavra, 1962a, b; Sizer and Jenkins, 1963; Hammes and Fasella, 1963; Jenkins and D'Ari, 1966c). These compounds, reacting preferentially with the protonated aldimine enzyme, form oximes and thiosemicarbazides respectively with enzyme bound PALP, greatly influencing the visual absorption spectra.

The monocarboxylic amino acids, L-alanine, L-serine and L-methionine also bind to the enzyme and are slowly transaminated (Jenkins, 1961a; Novogrodsky and Meister, 1964). The slow rate of transamination with alanine is apparently due in part to its low affinity for AAT (Harruff and Jenkins, 1976b), suggesting the involvement of both carboxyl groups in binding. This conclusion is supported by the observed potent inhibition of AAT by certain dicarboxylic acids. Those dicarboxylic acids that most nearly match the molecular geometry of the natural substrate are the most effective inhibitors (Haarhoff, 1969). The observation

that maleate inhibits AAT more strongly than fumarate has further defined the configurational requirement of cis carboxyl groups for effective binding (Jenkins et al., 1959a).

Mason (1959), studying the inhibition of kynureine transaminase by dicarboxylic acids obtained similar results. To explain these results he postulated the presence on the enzyme of two cationic groups capable of binding both carboxyl groups of both substrates and inhibitors. This hypothesis has been extended to AAT (Sizer and Jenkins, 1963).

The interaction of dicarboxylic acids may be studied by direct spectrophotometric titration. Addition of certain dicarboxylicacids, notably maleate and glutarate to aldimine ATT, causes a decrease in the absorption at 360 nm and an increase at 430 nm (Jenkins et al., 1959a). As a result of complex formation with dicarboxylic acids, the pK_a of the 430/360 nm transition is raised from ca 6.3 to 8.0 (Sizer and Jenkins, 1963).

Jenkins and D'Ari (1966c; 1966d; 1968) have studied the binding of glutarate to aldimine AAT. Their results have been interpreted to support a model of glutarate binding in which, at high pH ($>$8.0), one carboxyl group only is bound. In so doing it displaces a counterion. At low pH ($<$8.0) the second carboxyl group is also bound with a net displacement of two counterions. The dissociation constant of the enzyme-glutarate complexes decreases with decreasing pH. This pH effect, coupled with its abolition by a carbonyl-modifying reagent (thiosemicarbazide) that attacks the aldimine linkage (Jenkins and D'Ari, 1966c),

supported tentative assignment of the low pH binding site to the protonated internal aldimine nitrogen. The high pH enzyme-glutarate complex absorbs at 360 nm whereas the low pH complex absorbs at 430 nm because of the compulsory protonation of the aldimine nitrogen. Spectrophotometric fast-reaction studies on the binding of glutarate suggested that the formation of the high pH monodentate ligand was via the intermediate formation of the bidentate complex; the reaction occuring with the transient formation of an intermediate absorbing at 430 nm. These results imply the sequential formation of enzyme-substrate bonds, and that their simultaneous presence is not required for substrate specificity (Jenkins and D'Ari, 1966d; 1968).

Studies on dicarboxylic acid binding by using ^{19}F n.m.r. have similarly identified the low-pH binding site as the protonated aldimine (Martinez-Carrion et al., 1973). These authors studied the interaction of perfluoro-succinate by measuring the broadening of the ^{19}F n.m.r. signal and the chemical shift on binding to AAT. Band broadening, a measure of the tightness of binding, showed a pH variation. This parameter decreased with decrease in pH with inflexion points at pH 5.4 and pH 8.0. Under the condition used for these experiments i.e. deionized enzyme, it has been shown that the pK_a of the protonated internal aldimine is 5.4 (Cheng et al., 1971).

Ivanov and Karpeisky (1969) have suggested that the previously mentioned hypothetical site that binds the phenolic anion of PALP might be the low pH binding site. This postulate could certainly account for most of the observations on the low pH binding site. Addition of a

counterion (from a dicarboxylic acid) to this site would abolish the proposed effect of this group in depressing the pK_a of the internal aldimine. The pK_a would then rise to approximately 8.0, as observed. This would, however, have to be reconciled with the poor binding affinity that dicarboxylic acids have for the apoenzyme, (Martinez-Carrion et al., 1973).

Martinez-Carrion et al., (1973) have attributed the high-pH binding site to a histidine residue. The evidence for this lies in similar pH dependence and mutual exclusivety of anions and dicarboxylic acids suggesting that they occupy the same subsite. One anion-binding site has been identified as an essential histidine residue (Cheng and Martinez-Carrion, 1972; also see later). However, there is apparent confusion here, as Cheng and Martinez-Carrion (1972) characterised this anion-binding site with a pK_a of 6.2. Jenkins and D'Ari (1968) have also suggested that the pK_a of the high pH carboxylate binding site is greater than 11.

The binding of the substrate analogue α-methylaspartate is pH-independent in the absence of anions (Cheng and Martinez-Carrion, 1972). The observation that under identical conditions binding of dicarboxylic acids is pH dependent has led Martinez-Carrion et al., (1973) to propose that although the natural substrate and dicarboxylic acids are mutually exclusive they do not share the same subsites. Such observations illustrate the caution that is required in interpreting studies of dicarboxylic acid binding as a model for substrate carboxylate group binding.

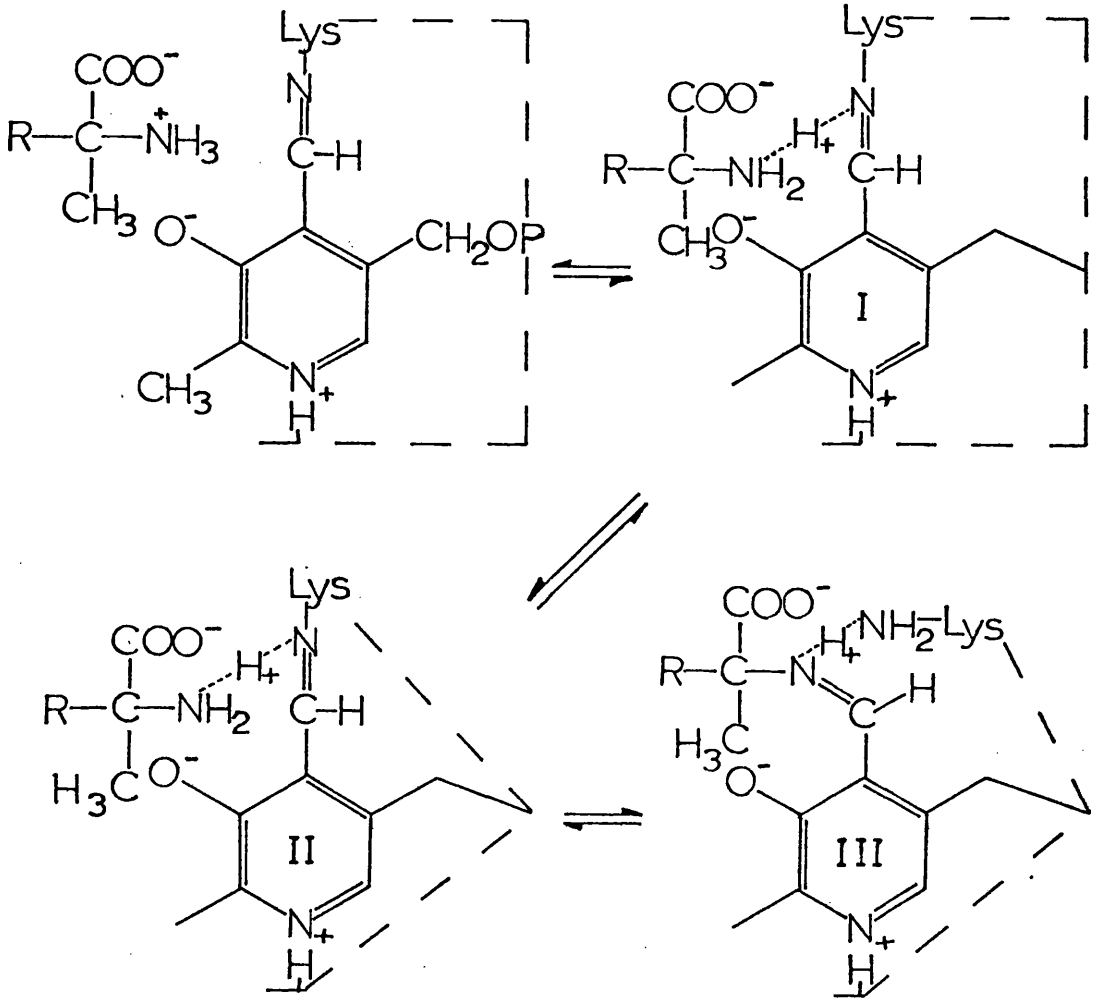
Studies with pseudo-substrates that more nearly match

the natural substrate have provided information on the formation of enzyme-substrate covalent complexes.

Such a substrate analogue is α-methylaspartate, which would be expected to bind to the aldimine enzyme but not to be transaminated because of its lack of an α-H atom. α-methylaspartate was found to be an inhibitor of the enzyme (Jenkins et al., 1959a). Its addition to aldimine enzyme caused marked changes in the absorption spectrum (Jenkins et al., 1959a; Fasella et al., 1966), and optical activity of the coenzyme (Fasella et al., 1966; Torchinsky and Koreneva, 1964a; Torchinsky, 1964; Martinez-Carrion et al., 1970a). These results were interpreted in terms of formation of a Schiff base between α-methylaspartate and enzyme-bound PALP. Confirmatory evidence for this was obtained by reduction of the enzyme-methylaspartate complex with sodium borohydride, and isolation of a pyridoxyl-methyl-aspartate complex (Malakhova and Torchinsky, 1965). The spectrum of the enzyme-methylaspartate complex has pH-independent spectral maxima at 362 n m and 430 n m This duplicity of spectral maxima has been proposed to suggest that the complex exists in at least two isomeric forms; protonated and unprotonated substrate-aldimine complexes interconverted by intramolecular proton transfer (Fasella et al., 1966; Hammes and Tancredi, 1967; Fonda and Johnson, 1970).

The kinetics of the binding of α-methylaspartate to aldimine AAT have been studied by stopped-flow and temperature-jump methods (Hammes and Haslam, 1968). Analysis of the results indicated a fast bimolecular step followed by two slower unimolecular steps. All six rate

Figure 6



Proposed mechanism for the reaction of α-methylaspartate with AAT, according to Hammes and Haslam (1968).

constants were estimated and a mechanism for the reaction proposed (Fig.6). A satisfactory explanation of the data, at low α-methylaspartate concentration, required the introduction of substrate activitation into the mechanism. The initial bimolecular step was seen as the formation of the non-covalent complex (Fig. 6,I) whereas the last step was thought to involve the formation of the covalently bound α-methylaspartate Schiff base (III). The second order rate constant for the initial bimolecular reaction was considerably slower than that in the case of the natural amino acids, so too presumably are the intermediate steps. The slowing down of reactions in this way with the corresponding facilitation of kinetic analysis, illustrates one of the possible advantages accruing from the use of pseudo-substrates for extracting information about individual steps in a complex rapid enzymic reaction.

The spectra of intermediates I and III were determined and each was found to have absorption maxima at 360 and 430 nm. This suggested that each complex might exist in two rapidly equilibrating conformers, possibly also involving intramolecular proton transfer. The intermediate II (Fig.6) was only present at all times in relatively small concentrations and its spectrum was not confirmed. II was suggested to be an isomer or a different conformational state of I or III, and is so designated (by changes in the dashed line representing protein) in Figure 6.

The thermodynamics of the interaction of α-methylaspartate with AAT have been studied (Hammes and Tancredi, 1967). The positive enthalpy change on binding was interpreted as resulting from the lower energy of the internal

Schiff base compared with that of the α-methylaspartate Schiff base; the latter being formed solely because of the favourable entropy change.

The threo and erythro-isomers of β-Hydroxy aspartate are slowly transaminated by AAT (Jenkins, 1961b). Of particular interest is the reaction of erythro-β-Hydroxy-aspartate. This substrate analogue reacts with aldimine AAT to form a mixture of intermediates with an intense absorption peak at 490 nm. The equilibrium kinetics of this interaction have been studied by Jenkins (1961b; 1964) and the absorption at 492 nm was assigned to the quinoid-type intermediate (cf. Fig. 3, Structure III). This complex was on the aldimine side of the rate limiting step, either side of which were roughly equal proportions of binary complexes at all pH values. A fast reaction kinetic study of AAT with this analogue was initially performed by Czerlinski and Malkevitz (1965). However a more extensive study has been reported by Hammes and Haslam (1969), superceding those initial results. By a combination of temperature jump, stopped flow and direct spectrophotometry, eleven relaxation processes were observed, ranging in time from $<10\mu s$ to >1 day. The two longest relaxation processes were concentration-independent and were attributed to keto acid product (oxaloglycolate) decomposition. The shortest relaxation time was assumed to be associated with a non-productive intermediate off the main pathway. The remaining processes (8) were evaluated on the basis of a sequential mechanism involving seven intermediates. Computer analysis allowed determination of seven out of the eight pairs of rate constants. The wavelength-dependences of the amplitudes of

the relaxation processes for the first three intermediates were comparable with those of the three relaxation processes observed with α-methylaspartate indicating similar structures for the corresponding intermediates. The rate constants associated with these steps were however larger than the corresponding values for α-methylaspartate. The fourth intermediate was assigned the quinoid-type structure, while the remaining intermediates with apparent spectral maxima around 330 nm were designated as ketimines and non-covalent aminic-enzyme keto-acid complexes. The rate-limiting step,occurring between the fifth and sixth intermediate, was thus assigned to the hydrolysis of (or a conformational change in) a ketimine complex. The above assignment of the rate-limiting step is in contrast to that suggested for the natural substrates and model systems, i.e. α-proton labilization (Banks et al., 1968b; Doonan et al., 1970).

The above work with β-hydroxy-aspartate suggests the existence in the enzymic reaction of at least three substrate-ketimine complexes. Information on the formation of substrate-ketimine complexes is not as readily available as on the formation of substrate-aldimine complexes, largely because of the lack of a suitable stable keto acid analogue.

Studies on the interaction of dicarboxylic acids with aminic AAT are also hampered, relative to those with the aldimine enzyme, by lack of a spectral change. Specificity studies have indicated that both carboxylate groups are involved in binding. α-keto dicarboxylic acids bind more strongly than do simple α-keto acids (Banks et al., 1963b). Steady state kinetics (Haarhoff, 1969; Velick and Vavra, 1962a, b), n.m.r. (Martinez-Carrion et al., 1973; Dybel et al.,

1972), and competitive effects on the binding of β-hydroxy-aspartate (Jenkins, 1964; Michuda and Martinez-Carrion, 1970) are methods that have been used to study the binding of dicarboxylic acids to the aminic enzyme.

From a consideration of the relative affinities of the aminic and aldimine forms of the enzyme for various dicarboxylic acids Fasella and Turano (1970) have suggested a small difference in the geometry and charge distribution between the two enzyme forms. Maleate and glutarate bind equally well to the aldimine enzyme (Jenkins et al., 1959a; Michuda and Martinez-Carrion, 1970) whereas only maleate binds well to the aminic enzyme (Jenkins, 1964). These differences are also reflected in the behaviour of the natural keto-acid substrates; oxaloacetate binding to the aminic enzyme more strongly than does 2-oxoglutarate, the reverse being true for the formation of the aldimine enzyme-keto-acid abortive complex (Velick and Vavra, 1962; Henson and Cleland, 1964).

Using an extensive steady state kinetic analysis of the inhibition of AAT by a homologous series of dicarboxylic acids, Haarhoff (1969) examined the binding affinity of these compounds to the aminic enzyme. He concluded that the aminic enzyme has little affinity or specificity for the dicarboxylic acids, relative to the aldimine enzyme. However, these results more probably reflect the greater affinity of the aminic enzyme for competitive anions (Cheng et al., 1971). Both succinate and perfluorosuccinate have been shown by n.m.r. to bind more strongly to the aminic enzyme than to the aldimine enzyme (Dybel et al., 1972; Martinez-Carrion et al., 1973); this is also true of

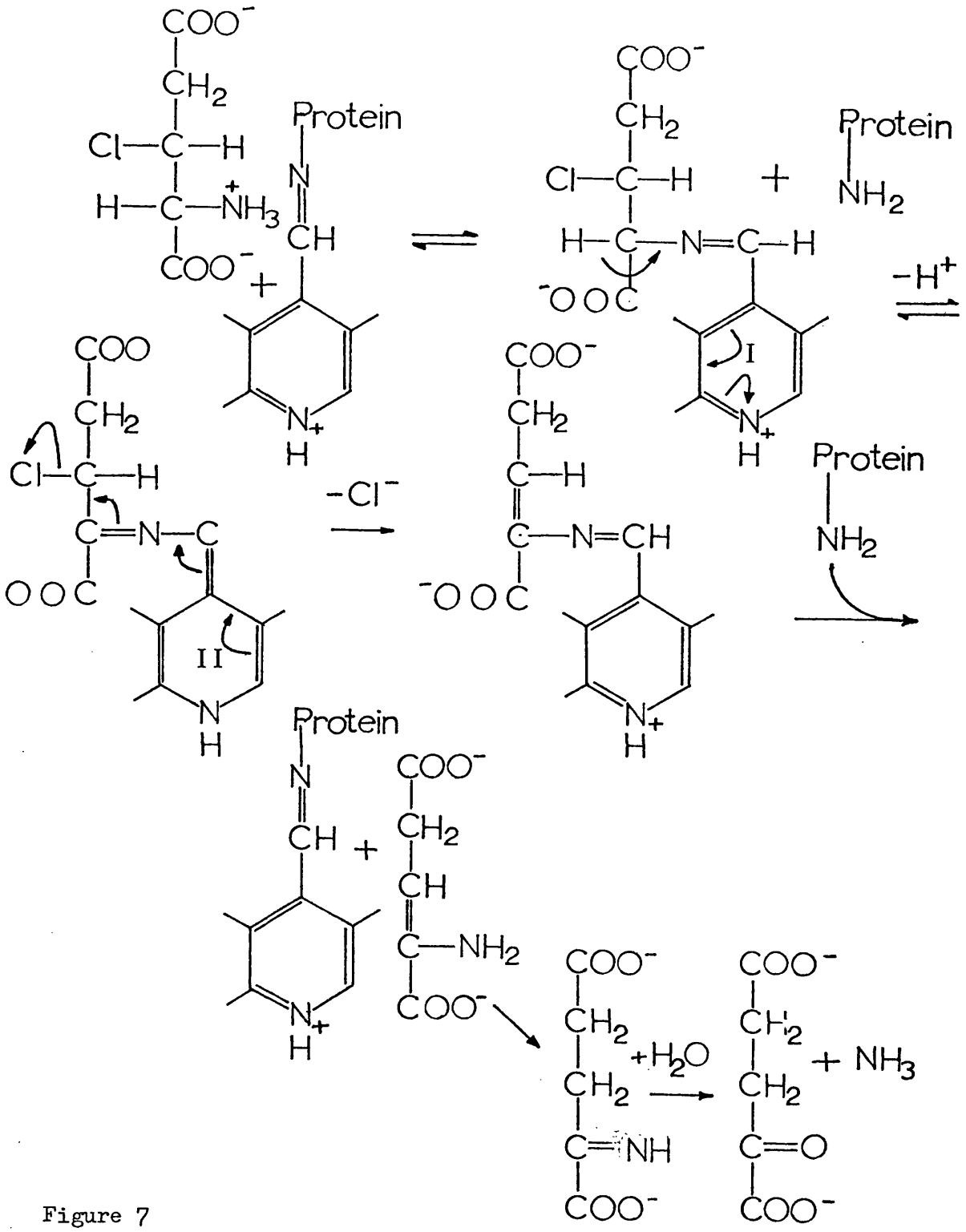


Figure 7

Tentative scheme for the interaction of AAT with threo-β-chloro-glutarate, according to Antonini et al., (1970).

glutarate (Michuda and Martinez-Carrion, 1970).

AAT is known to catalyse atypically the α,β-elimination of certain quasi-substrates with strongly electronegative β-substituents. In the presence of catalytic amounts of aldimine AAT the threo and erythro isomers of β-chloroglutamic acid undergo such a reaction producing Cl^-, NH_4^+ and 2-oxoglutarate (Manning et al., 1968). The reaction of threo-β-chloroglutamate has been studied by stopped-flow methods (Antonini et al., 1970), which indicated that the reaction involves the initial rapid formation of a spectroscopically distinct (λ_{max} 350 nm) enzyme-substrate complex followed by a slow breakdown of this complex with the eventual formation of products. The initial rapid reaction was studied in isolation and a structure was assigned to the enzyme-substrate complex (Fig.7,I). A mechanism for the reaction was suggested (Fig. 7), which was based on that suggested by Braunstein (1960) for pyridoxal catalysed β-elimination reactions. The rate limiting step was deduced to be α-proton labilization, (Fig. 7,I → II), a step common to both β-elimination and transamination. The thermodynamic and kinetic parameters for the formation of (I) were comparable with those obtained for α-methylaspartate and reflect that already discussed for α-methylaspartate. However, as pointed out by the authors, intermediates undetected prior to (I) could complicate these comparisons.

Kun et al., (1960) reported what would seem to be a similar β-elimination reaction. On incubation of β-fluorooxaloacetate with AAT and an amino donor, ammonia and oxaloacetate were formed and the reaction was suggested to occur via the intermediate formation of β-fluoro-aspartate followed

by dehydrofluorination and deamination. The authors were unable either to isolate β-fluoro-aspartate from the incubation mixtures or to synthesize the compound chemically. The α-β-elimination reactions of β-chloro-L-alanine and bromopyruvate are described below.

Identification of functional amino acid residues at the active site has been attempted. Residues involved in coenzyme binding (already discussed) and those residues believed to be involved in catalysis of the tautomerization step have received most attention. Histidine, lysine and cysteine residues have all been proposed as being responsible for substrate α-proton labilization.

Active site histidine residues have been extensively studied by Martinez-Carrion and associates. Dye-sensitized controlled photo-oxidation of AAT resulted only in the loss of two histidine residues per monomer. Kinetic analysis indicated that the concomittant loss in enzyme activity was attributable to the loss of one essential imidazole group (Martinez-Carrion et al., 1967b). The structure of the photo-oxidised enzyme which was able to bond coenzyme was not grossly altered (Martinez-Carrion et al., 1970b) and the resulting holoenzyme was also capable of forming aldimine and ketimine intermediates with amino and keto substrates respectively (Martinez-Carrion and Peterson, 1969; Peterson and Martinez-Carrion, 1970).

Photo-oxidised enzyme was defective in its ability to form the semi-quinoid intermediate (λ_{max} 490 nm) with erythro-β-hydroxy-aspartate, and a reduced rate of substrate α-proton exchange compared to that of the natural enzyme was observed. The role of proton acceptor was pro-

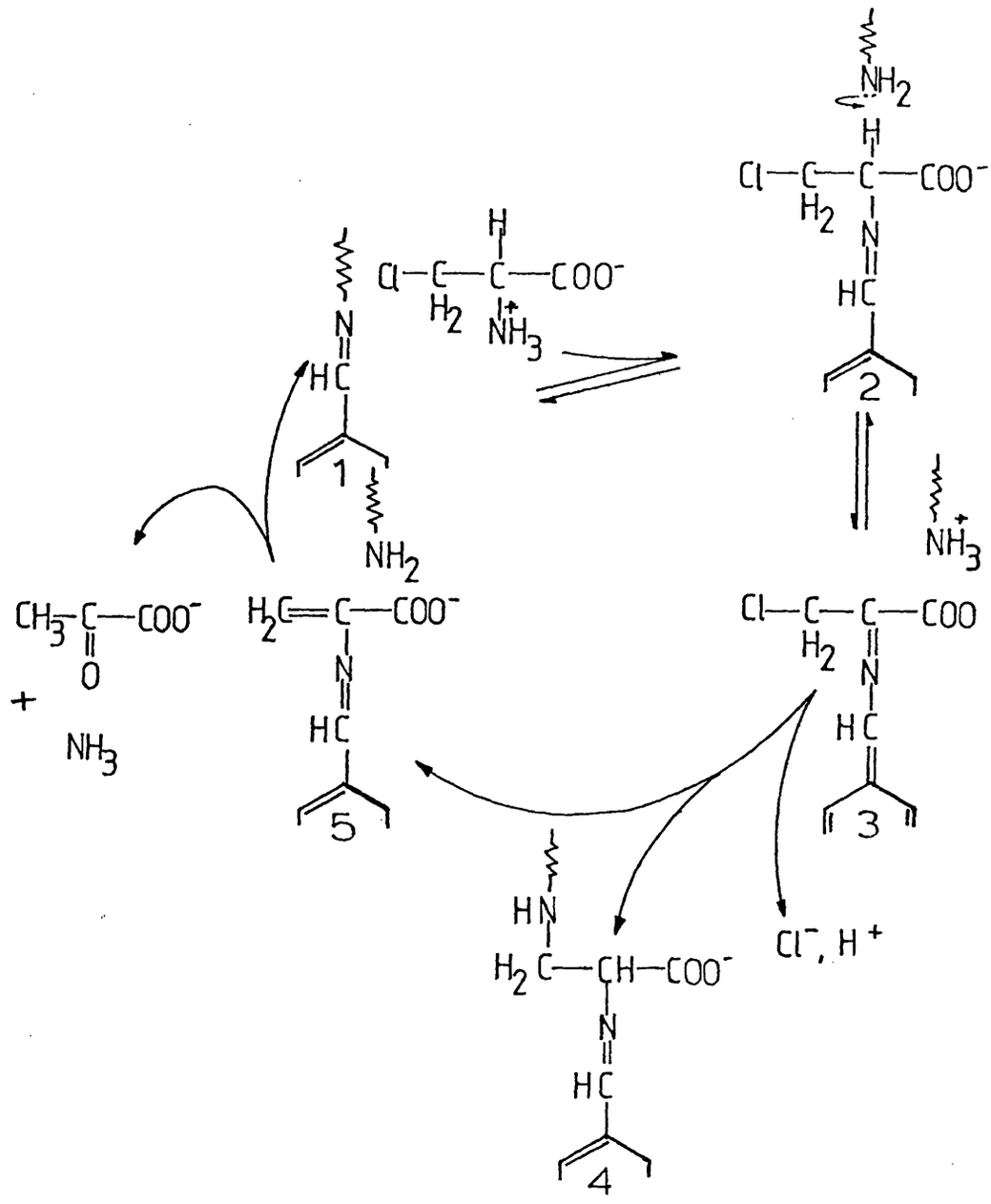


Figure 8

Scheme for the interaction of β-chloro-L-alanine with aspartate aminotransferase from Morino et al., (1974).

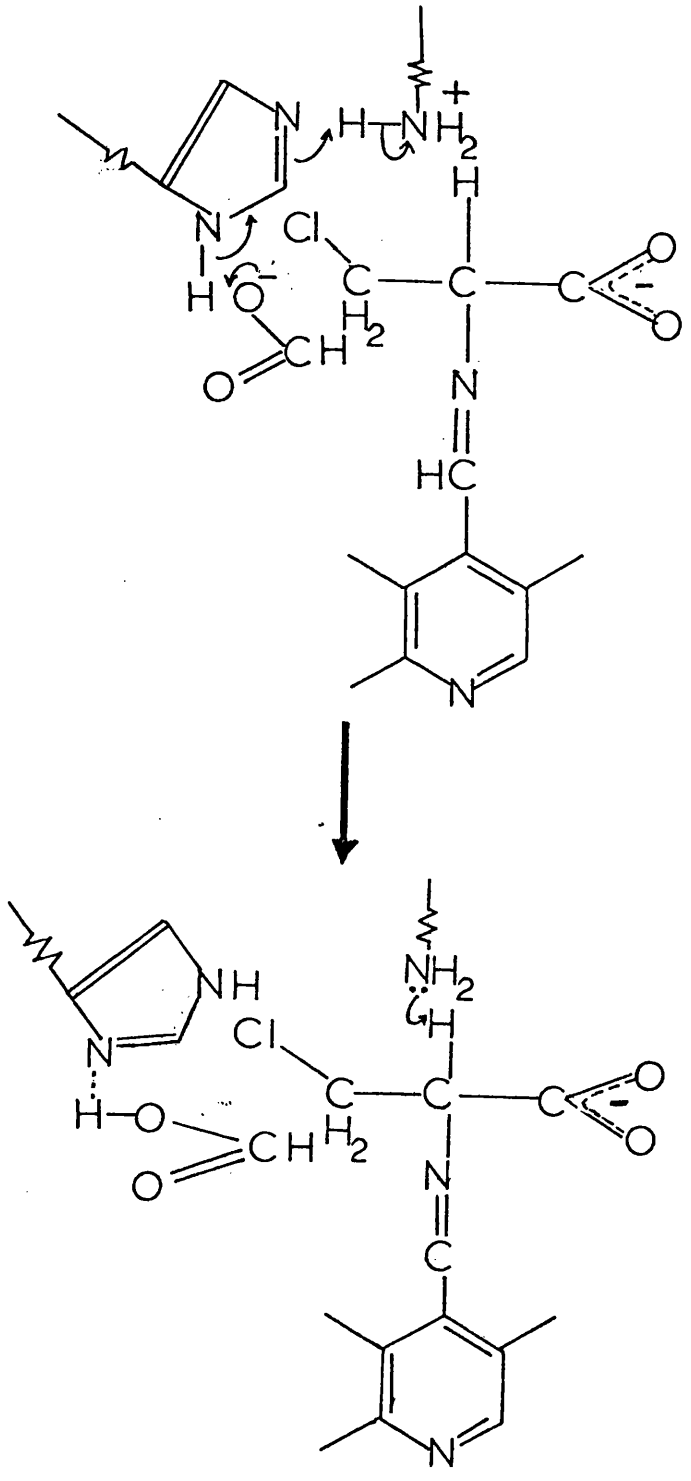
1, pyridoxal form of AAT; 1→2 Transaldimination; 2→3 Deprotonation; 4, inactive enzyme; 5, enzyme-α-amino acrylate complex.

posed for this vital histidine. The involvement of a histidine residue here is an attractive idea as Bruice and Topping (1963) have demonstrated imidazole catalysis of the tautomerization step in a model system. After photo-oxidation, lack of anion interference with substrate and pseudo-substrate binding suggested that one or other of the histidines might be the anion binding site.

Other evidence has suggested lysine as the residue responsible for the role of proton acceptor. β-chloro-L-alanine undergoes α,β-elimination in the presence of AAT with concommitant inactivation of the enzyme resulting from the covalent modification of $lysine_{258}$ (Morino and Okamoto, 1972; 1973) (Fig. 8). The obvious corollary is that this lysine residue is in close proximity to the bound substrate molecule and in view of the involvement of α-proton abstraction in both transamination and α,β-elimination it was suggested the lysine might carry out this function.

The above results thus present two apparently conflicting viewpoints on the nature of the amino acid responsible for α-proton abstraction. However, studies on the formate enhancement of both α,β-elimination and inactivation of AAT by β-chloro-L-alanine have led to the suggestion of a reconcillatory mechanism (Morino et al., 1974). It was proposed that a formate ion binds to a discrete subsite on the enzyme (i.e. as histidyl residue) which would normally be occupied by the distal carboxyl group of the substrate. This binding would set up a charge relay system between the formate ion, the hypothetical imidazoyl group and the adjacent lysyl residue, enhancing the proton abstracting properties of the latter (Fig. 9). A similar mechanism

Figure 9



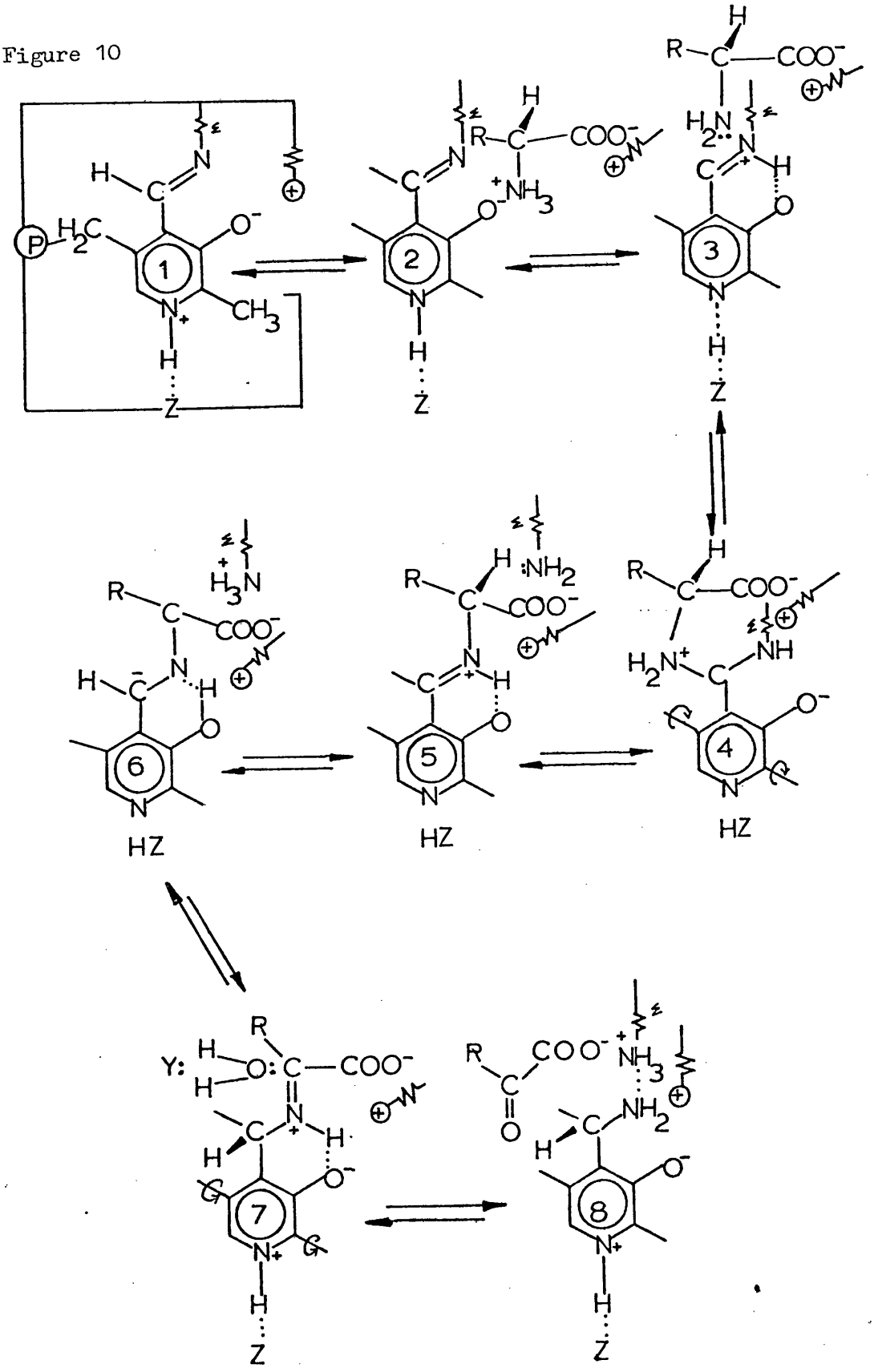
Suggested mechanism for the action of formate in the α, β-elimination of β-chloro-L-alanine, from Morino et al., (1974).

was envisaged for the natural substrates. In support of this mechanism it was subsequently demonstrated that photo-oxidised AAT (with the presumed loss of a histidine residue) was fully active in the α,β-elimination of β-chloro-L-alanine, but did not demonstrate a formate enhancement of α,β-elimination (Yamasaki et al., 1975). Turano et al., (1966; 1968) using the bifunctional reagent 3-bromo-propionyl-chloride have demonstrated the close proximities of a histidine and a lysine residue at the active site of AAT.

The role of α-proton acceptor for a thiolate anion has been proposed on the basis of studies of the inactivation of AAT by bromopyruvate (Okamoto and Marino, 1973). The mechanism of inactivation proposed was similar to that suggested for inactivation of AAT by β-chloro-L-alanine, except that in this case, a cysteinyl residue was covalently modified. Birchmeier and Christen (1974) have suggested, however, that rather than acting as an affinity label, under certain conditions, bromopyruvate can act as an alkylating reagent in a syncatalytic modification reaction. Bromopyruvate was shown to modify cysteine-390, a residue syncatalytically modifiable by a variety of reagents. The degree of inhibition of AAT on modification of this residue is dependent both on the size and charge of the substituent, indicating that the residue is located near the active site only and not essential (Birchmeier et al., 1973a).

In order best to interpret the available evidence on the mechanism of enzymic transamination a dynamic model has been developed by Karpeisky and his associates (Karpeisky and Ivanov, 1966; Braunstein et al., 1968; Ivanov and Karpeisky 1969). This model attempts to explain the cata-

Figure 10



Dynamic model of enzymic transamination.

lytic mechanism in terms of the sequential transitions in electronic and geometrical patterns of the enzyme-substrate intermediates. An essential central feature of this model is the postulate that the coenzyme moiety undergoes a reversible reorientation in the enzyme in order to produce the correct geometrical relationship of substrate, cofactor and enzyme for the transaldimination and subsequent steps. This rotation of the chromophore was suggested to account for the observed decrease in its optical activity on addition of the substrates.

The dynamic model is outlined in Fig. 10. The state of the coenzyme prior to the addition of substrate (1) has already been discussed. It is suggested that on formation of the initial non-covalent complex (2), the substrate α-carboxyl group binds to that positively-charged group normally associated with the ionized phenolic group. Loss of this ion-pair causes a rise in pK_a of the internal aldimine. Neutralization of the α-carboxyl reduces the pK_a of the substrate amino group. Thus a proton is transfered from the substrate amino group to the coenzyme (3). The protonated Schiff base is then liable to nucleophilic attack by the lone pair on the substrate amino group.

Considerations on the stereochemistry of nucleophilic addition and the requirement for the amino group formyl carbon distance to close from >3.5 Å to the length of the C-N bond (i.e. 1.5 Å) lead to the proposal of the reversible reorientation of the coenzyme. Karpeisky and his associates suggest that the coenzyme rotates through an angle of approximately 40° about the axis formed by the 2' and 5' carbons. This is permitted by rupture of the

pyridine nitrogen-hydrogen bond with group Z, itself resulting from the preceding protonation of the chromophore and associated lowering of the pK_a of the ring nitrogen. With the pyridine ring thus rotated, covalent bonding of C-4' to the substrate N atom produces the tetrahedral intermediate (4). The equivalent basicities of the two nitrogens attached to C-4' permit proton translocation to the lysyl ε-nitrogen. The positive charge on the lysyl ε-nitrogen facilitates its elimination and completion of the transaldimination step (5). The next step involving the prototropic aldimine-ketimine rearrangement was originally proposed to be promoted by 2 functional groups; one accepting the C-α proton and another protonating C-4'. In the light of Dunathan's work (1971) suggesting a cis transfer for this step, Braunstein (1973) has modified the dynamic model, these steps being carried out by the ε-NH_2 of lysine with the transient formation of a quinoid type intermediate (6), the C-4' atom acquires tetrahedral configuration on protonation (7) and so permits rotation of the coenzyme to its original plane. Reprotonation of the pyridine nitrogen increases the acidity of the phenolic hydroxyl facilitating the next step by transfer of its proton to the amino nitrogen. Nucleophilic addition of a molecule of water at C_α produces, via the transient hydrated ketimine (7), the α-oxoacid and pyridoxamine (8).

It was further suggested that in the pyridoxamine form of the enzyme the amino group is maintained in its reactive unionized form. This was brought about by the proximity of two cationic groups, the ε-amino group of

lysine and the previously mentioned cationic protein group.

In the article of Ivanov and Karpeisky (1969) the model, presented in greater detail, was used to explain some further properties of AAT. Experimental verification of the model has also been attempted (Ivanov and Karpeisky, 1969; Bocharov et al., 1974; Mora et al., 1972; Kogan et al., 1974; Tumanyan et al., 1974).

Work previously carried out in this laboratory has demonstrated the suitability of difluoro-oxaloacetate as a probe of the reactions of the aminic form of the enzyme. Steady state kinetic analysis of the interaction of difluoro-oxaloacetate with AAT indicated that the fluoroanalogue showed a strong interaction with aminic enzyme (Briley et al., 1973; Briley et al., 1977a), and at higher concentration than used in the steady state kinetic analysis, was also shown to form an abortive complex with aldimine enzyme. This interaction was studied by ^{19}F fluorine nuclear magnetic resonance (Briley et al., 1977b). Variation of the ^{19}F chemical shift and line width, arising from bound ligand, indicated tighter binding with decreasing pH. Two inflection points were observed in this curve, one at pH 8.0 and one at pH 5.4. This was interpreted in terms of the binding of both carboxyl groups of difluoro-oxaloacetate requiring successive protonation of two groups on the enzyme. The group responsible for the inflexion at pH 5.4, absent in studies on the binding of this ligand to the apoenzyme was tentatively suggested to be the protonated aldimine nitrogen. This is in general agreement with previously discussed results.

The reaction of difluoro-oxaloacetate with aminic

enzyme was studied in detail (Briley *et al.*, 1977c). The addition of difluoro-oxaloacetate to aminic enzyme results in the initial rapid shift of the absorption maximum from *ca* 332 to 328 nm followed by a slow shift to 360 nm, indicating transamination. Transamination of difluoro-oxoacetate to a substance having a ^{19}F n.m.r. spectrum attributable to the cognate amino acid, difluoro-aspartate, was demonstrated. Analysis of the concentration-dependence of the 332→360 nm transformation indicated the rapid formation of at least one binary complex coupled with its slow decay. The kinetic parameters for these reactions were estimated from initial rates and from the determination of the pseudo-first order rate constant for the 332→360 nm transformation.

The rapid spectral change 332→328 nm was studied by stopped flow spectrophotometry. Analysis of these results suggested that the formation of the spectrally distinct species (λ_{max} 328) was via a spectrally indistinguishable (λ_{max} 332) intermediate. The λ_{max} 328 nm intermediate was tentatively identified as a substrate ketimine intermediate or its precursor, the tetrahedral carbinolamine.

Fasella and Turano (1970) reflecting on the interest there would be in a study of the pH dependence of the formation of ketimine enzyme substrate complexes stated that as yet none had been performed. A study of the pH dependence of the kinetic parameters for the 332→360 nm transformation was therefore carried out in the presently described work. Such a study constitutes part 1 of the studies on AAT presented here.

Consideration of the effects that double substitution of fluorine will impart on aspartate suggested that difluoro-aspartate might prove to be an interesting substrate analogue with which to probe the reactions of the aldimine form of AAT. The preparation properties and interactions of difluoro-aspartate with AAT constitute part 2 of this thesis.

Materials and Methods

Materials

Difluoro-oxaloacetic acid and diethyl difluoro-oxaloacetic acid were prepared as described by Briley et al., (1977a). Triethanolamine hydrochloride and L-cysteine sulphinic acid were obtained from Sigma, (London); malate dehydrogenase, lactate dehydrogenase and NADH from Boehringer Corporation, (London); sodium $[^3H]$ -borohydride (332mCi/mM) from the Radio Chemical Centre, (Amersham); sodium cyanohydridoborate and chloro-difluoro-acetic acid from Aldrich; Hydroxylapatite (Bio-Gel HTP), AG 1-X8 anion exchange resin, and AG 50-W-X8 cation exchange resin from Bio-Rad Lab. Ltd.. Pig hearts were supplied by Scott-Bowyers Ltd., Trowbridge, Wiltshire. All other laboratory reagents were obtained from BDH Chemicals Ltd., Poole, Dorset.

Methods

Standard assay of enzyme activity.

Enzyme activity was routinely measured by the method of Banks et al., (1968a). The method relies on the spectrophotometric estimation of oxaloacetate production at 260nm under conditions where the enolisation of oxaloacetate is not partially limiting (Banks et al., 1963a). In a 1cm cuvette (3ml) containing 0.1M-triethanolamine buffer, pH 7.5, 64.5mM-L-aspartate and 6.85mM-2-oxoglutarate at 25°C the reaction was initiated by the addition of a small aliquot of enzyme. The unit of activity is defined as that amount of enzyme which causes a rate of increase in absorbance at 260nm of 0,1 absorbance unit per minute.

Protein concentration.

Protein concentration in crude preparations was estimated using the 215:225nm peptide bond absorption method of Waddel (1956). Protein concentration in purified enzyme was estimated from 280nm absorbance (Banks et al., 1968a).

Preparation of aspartate aminotransferase (AAT).

The aldimine form of cytoplasmic AAT was prepared

from fresh pig heart ventricles as described by Jenkins et al., (1959a) except that succinate buffer was used instead of maleate buffer for the initial extraction and heat treatment (Turano et al., 1964). In addition 1mM-2-oxoglutarate and 0.1mM-dithiothreitol were included in all buffers. The method of preparation is outlined in Table 1i . Table 1ii,illustrates the results of a typical preparation which yielded 442mg of AAT with a specific activity of 721u/mg.

Preparation of α-aspartate aminotransferase.

The above preparation of AAT gives a mixture of the α, β, and γ subforms. The α-subform was prepared from this mixture by the method of Martinez-Carrion et al., (1967a). In a typical prèparation AAT (200mg; specific activity 710u/mg in 0.02M-sodium acetate buffer (8.5 ml)), pH 5.33 (all buffers utilized contained 0.1mM-DTT) was applied to a column of CM Sephadex C50 (50cm x 2cm) equilibrated with the same buffer. The α-subform of AAT was eluted as the last fraction resulting from the application of a linear gradient of eluent (500ml, 0.06M (pH5.33) - 0.11M (pH 5.41)). Enzyme thus prepared (85mg) had a specific activity of 910 u/mg with a A_{430}/A_{340} ratio of 2.09 in 100mM-pyrophosphate buffer, pH 5.5. Under identical conditions the unpurified enzyme typically exhibited a A_{430}/A_{340} ratio of 1.46. Where this form of the enzyme is used for experimentation this will be stated.

Table 1i.

Purification procedure for AAT.

Pig heart ventricles (2Kg) were washed, diced and homogenised in 3 portions with 0.05M-succinate buffer pH 6.0 containing 5mM-EDTA, 0.1mM-DTT, (3 x 1.5l) in a gallon Waring blender at full speed for 1 minute.

↓

The homogenate was heated with stirring to 72-73°C and maintained at this temperature for 25 mins.. At 65°C 40mM-2-oxoglutarate (200ml) pH 6.0 was added. The homogenate was cooled to 10°C, filtered and adjusted to contain 0.1mM-DDT.

↓

Assay Step 1 ←

↓

Ammonium sulphate was added to 50% saturation and the mixture was centrifuged (3,000xg , 1h).

↓

Assay Step 2 ←

↓

Ammonium sulphate was added to the supernatant to 67% saturation and the precipitated protein (3,000xg ,1h) was collected, and dissolved in the minimum volume of succinate buffer (ca 50ml) and dialysed against 2l of running distilled water (containing 1mM-2-oxoglutarate and 0.1mM-DTT).

↓

Assay Step 3 ←

↓

Table 1i continued

The non-diffusable material was concentrated to ca 20ml by vacuum dialysis, brought to 0.02M phosphate, pH 6.8, with addition of 2M-phosphate buffer, pH 6.8 and clarified by centrifugation (5,000xg , 30 mins.).

↓

The supernatant was chromatographed on a column (40cm x 2.5cm) of hydroxyl-apatite and developed with 40mM-phosphate buffer, pH 6.8. AAT activity was eluted with 80mM-phosphate buffer, pH 6.8. Fractions containing AAT activity were combined and brought to 2.5mM with 2-oxoglutarate, pH 6.8.

↓

Assay Step 4 ←

To the combined fractions ammonium sulphate was added until the first permanent precipitate was formed. The mixture was centrifuged (5,000xg, 30 mins.) and a further 55g/l ammonium sulphate added. The precipitate was collected, dissolved in the minimum amount of double distilled water containing 0.1mM-DTT and dialysed against 2 x 5l of the same.

↓

The dialysate was freeze dried and the lyophilysate was stored over phosphorous pentoxide under vacuum at 5°C until required.

↓

Assay Step 5 ←

Table 1ii. Purification results for AAT.

Step	Volume ml	Total Protein mg	Total Activity U.	Specific Activity U./mg	Purification factor	Yield %
1	5440	51000	5243000	10.2	(1)	(100)
2	6220	-	469030	-	-	89.5
3	174	4070	440300	108	10.6	84.0
4	400	702	357800	510	50.0	68.2
5	-	442	318682	721	70.7	60.8

Preparation of the aminic form of aspartate aminotransferase.

The aminic form of AAT was prepared from aldimine enzyme by the method of Jenkins and D'Ari (1966a). Conversion was effected by the addition of a three-fold molar excess of cysteine sulphinate to the enzyme in 20mM-pyrophosphate buffer. Excess reagents and products were removed by passage through a column (50cm x 1cm) of Sephadex G25, equilibrated and eluted with the above buffer. The protein peak (monitored at 280nm) was collected and the specific activity was determined.

Estimation of the concentration of active sites in AAT samples.

The concentration of purified enzyme sites was estimated from the visual absorption spectrum of the enzyme. Extinction coefficients at the various wavelengths and at the various pH values of the two forms of the enzyme were estimated from the known extinction coefficients at pH 8.2 (Torchinsky et al., 1968).

Spectrophotometric measurements.

Unless otherwise stated all spectrophotometric measurements were performed in a Pye Unicam SP 1800 spectrophotometer at $25^{\circ}C \pm 1^{\circ}C$.

Results and Discussion: Part 1

The pH Variation of the Slow Reaction of the Aminic Form of Aspartate Aminotransferase with Difluoro-oxaloacetate.

As stated previously difluoro-oxaloacetate reacts rapidly with aminic-AAT to form an enzyme-substrate complex with λmax 328nm. This complex is then slowly transformed to yield the aldimine form of AAT and difluoro-aspartate. The reaction can be interpreted under the following scheme:-

$$E' + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \ldots(1)$$

λmax 332 $\qquad$ λmax 328 $\qquad$ λmax 360

(where E', aminic-AAT; S, difluoro-oxaloacetate; ES, enzyme-difluoro-oxaloacetate complex; E, aldimine enzyme; P, difluoro-aspartate), and can be conveniently followed by observing changes in absorption at 360nm.

All available evidence indicates that difluoro-oxaloacetate behaves, with respect to AAT, in a manner qualitatively similar to that of the natural keto substrates (Briley et al., 1977a, b and c). It was therefore felt that the above reaction might provide a suitable model with which to study the pH variation of the association of keto-acids with aminic-AAT and to provide valuable information on the formation of enzyme-substrate-ketimine complexes.

The slowness of the reaction coupled with the requirement of its observation necessitates the use of

high enzyme concentration, such that a situation arises where the concentration of aminic enzyme is comparable to the dissociation constant of the difluoro-oxaloacetate complex. Therefore in order to obtain a measurable difference in the rate of reaction, difluoro-oxaloacetate concentrations used for the study had also to be comparable to the concentration of the enzyme.

The assumption, implicit in the Michaelis-Menten treatment of such a system, that the total substrate concentration is essentially unaffected by the enzyme, is no longer tenable. In order to determine the kinetic parameters an alternative method was used. The method as described by Smith et al., (1977)(Smith, 1978) is outlined below:-

Assuming steady state in [ES] of equation (1)

$$\frac{d[ES]}{dt} = [E'][S]k_1 - [ES](k_{-1} + k_2) = 0$$

$$\text{let } K_m = \frac{k_{-1} + k_2}{k_1}$$

$$\text{then } [ES] = \frac{[E'][S]}{K_m}$$

Initial concentrations of aminic enzyme and difluoro-aspartate are [Et] and [St] respectively.

$$\text{then } [ES] = ([Et] - [ES])([St] - [ES])/K_m$$

$$\text{therefore } [ES]^2 - [ES]([Et] + [St] + K_m) + [Et][St] = 0 \quad ...(2)$$

The initial rate of reaction (v) is obtained at various values of [St] . The quadratic equation in [ES]

(equation (2)) is solved for assumed values of K_m.

Since $v = k_2 [ES]$

The correct value for K_m is that value which minimizes the sum of squares of residuals of the data points about the line in a plot of v versus [ES] which passes through the origin. k_2 is the slope of this plot.

The experimental procedure is as follows. A 1cm semi-micro quartz cuvette contained aminic-AAT (for concentration see legend to Figure 11) in 20mM-pyrophosphate buffer (1ml) and the reaction was initiated by the addition of a small aliquot ($\leq 10\ \mu l$) of a 40mM-difluoro-oxaloacetate stock solution.

The initial velocity (v, $uM.m^{-1}$) was determined from the initial rate of increase in absorbance at 363nm by making use of the difference in extinction between aminic and aldimine enzyme (see Methods; Smith, 1978).

To avoid the introduction of extraneous anions the pyrophosphate buffer was prepared by the titration of a degassed 20mM-tetra-potassium pyrophosphate solution with Biorad AG-50W-XS ion exchange resin (H^+ form).

The variation of initial rate (v) with difluoro-oxaloacetate concentration, for the various pH values, is presented in Figures 11i(a)——11v(a).

The complementary plots of initial rate (v) versus

computed [ES] , obtained from the best assumed value of K_m, are presented in Figures 11i(b) —— 11v(b). The kinetic parameters obtained are sumarized in Table 2.

Figure 11

Determination of the pH variation of the kinetic parameters for the slow reaction of difluoro-oxaloacetate with aminic aspartate-aminotransferase.

(a) Plots of initial rate (v) versus initial difluoro-oxaloacetate concentration [St] , for the pH values:-
i, 9.0 ; ii, 8.4 ; iii, 7.4 ; iv, 6.4 ; v, 5.4 ;
and the enzyme concentrations 27.5 μM; 28.2 μM;
18.7 μM; 24.8 μM; and 34 μM; (sites) respectively.

(b) Plots of initial velocity (v) versus computed [ES] (complimentary to (a)). [ES] was computed from the knowledge of [Et] and [St] and the best assumption of the value of K_m as described in text.

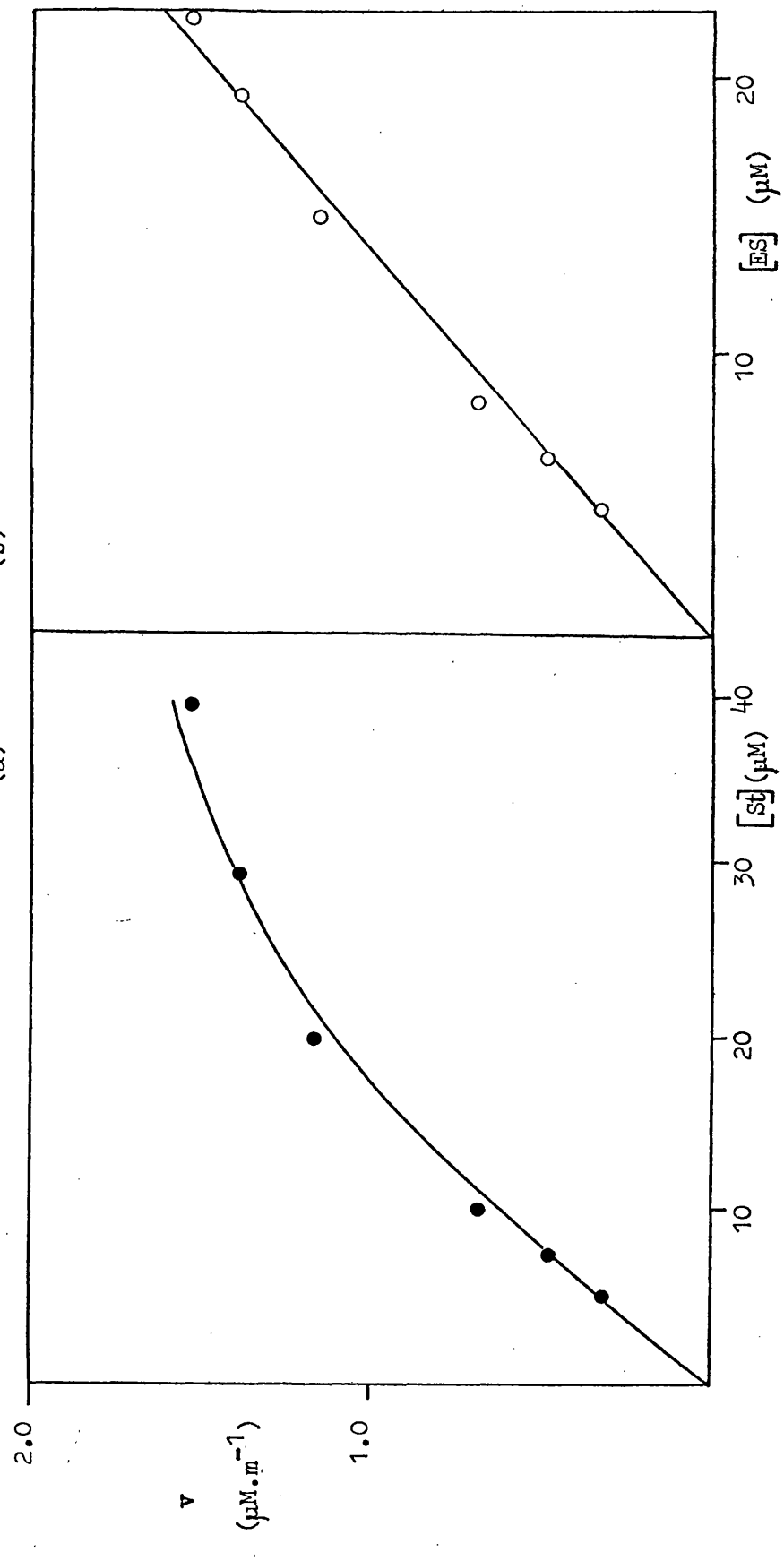


Figure 11 i. pH 9.0

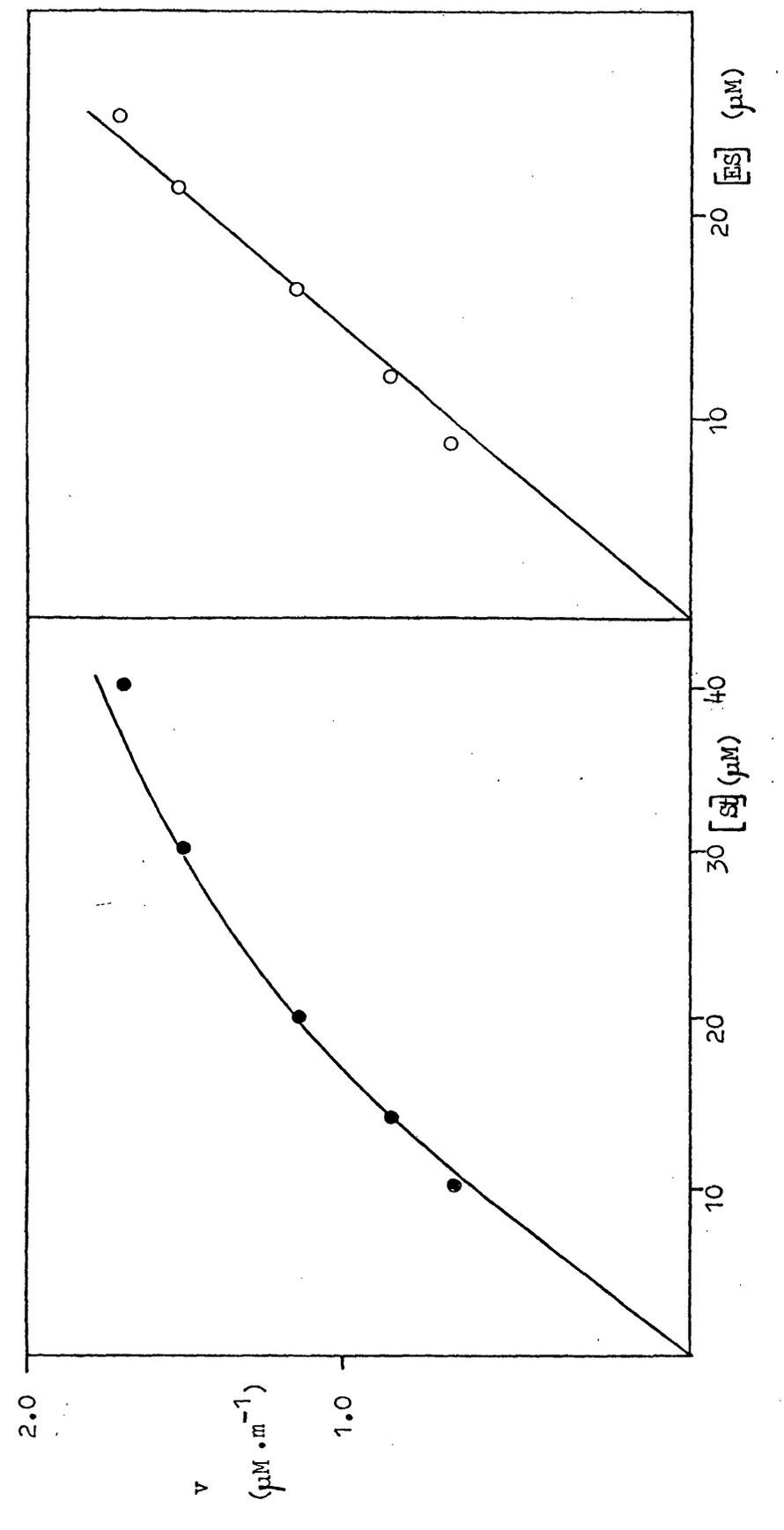


Figure 11i. pH 8.4

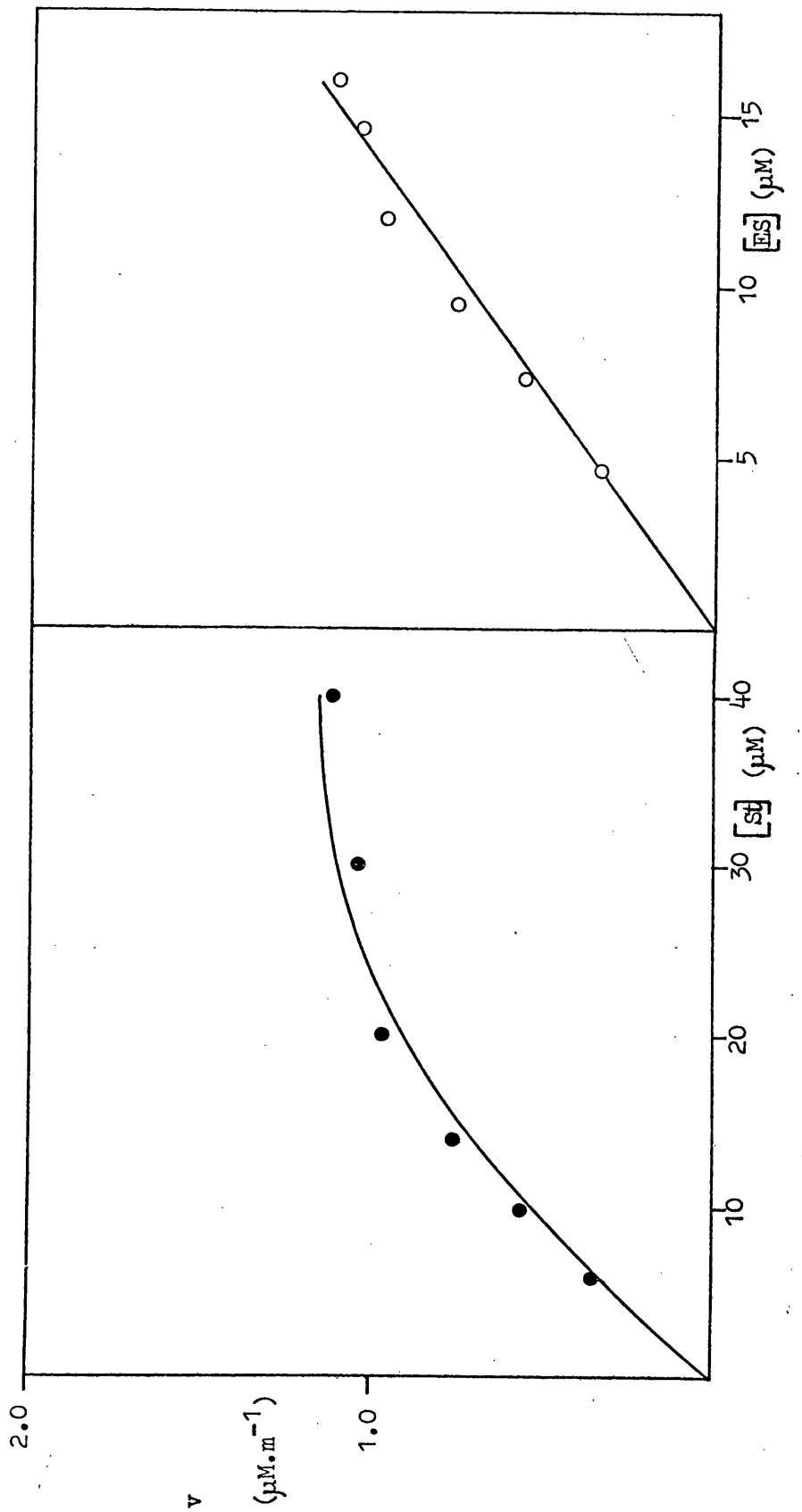


Figure 11ii. - pH 7.4

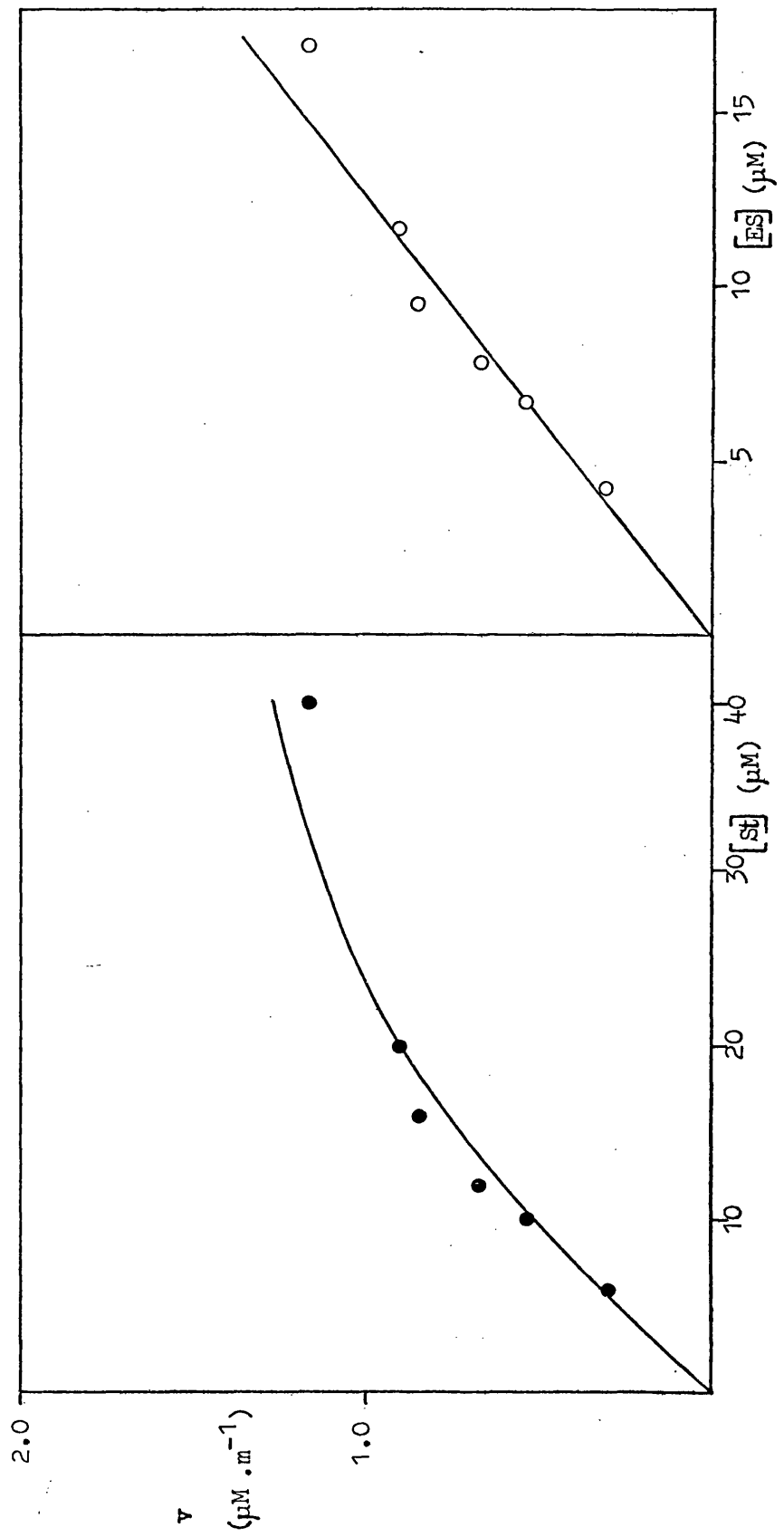


Figure 11iv. pH 6.4

Figure 11v. pH 5.4

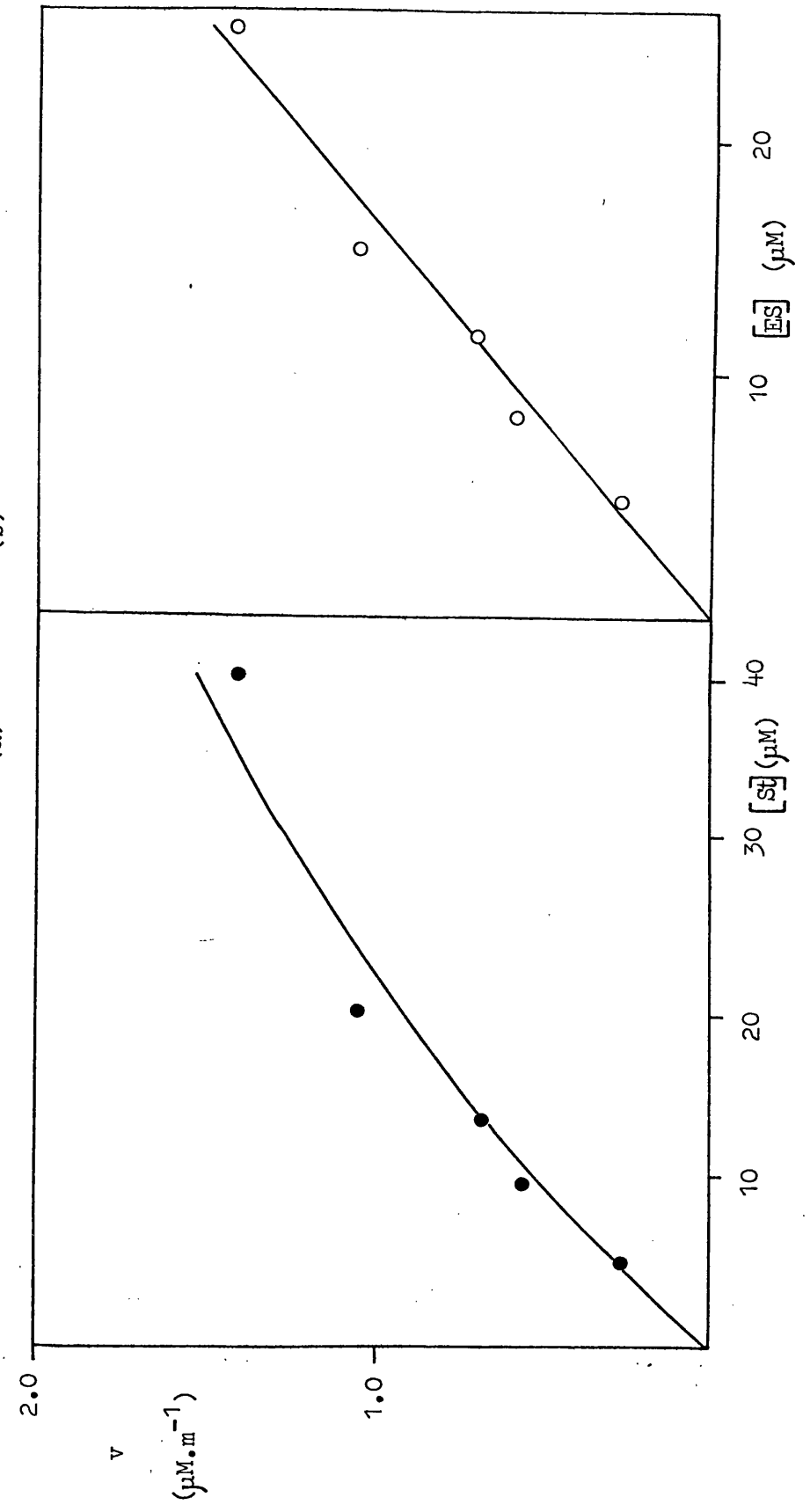


Table 2

Variation in the kinetic parameters K_m and k_2, for the slow reaction of aminic aspartate aminotransferase with difluoro-oxaloacetate, with pH.

pH	K_m μM	k_2 $s^{-1} \times 10^3$
9.00	4.0	1.19 ± 0.09*
8.40	3.7	1.19 ± 0.03
7.40	4.3	1.20 ± 0.05
6.40	3.9	1.05 ± 0.15
5.40	5.2	1.02 ± 0.33

* Standard error of the line in plots of initial rate (v) versus computed [ES] . Figure 11i(b)—— 11v(b).

From Table 2 it can be seen that the determined kinetic parameters are essentially invariant with pH, suggesting that no solution protons participate either in determining the association of aminic-enzyme with difluoro-oxaloacetate or its subsequent transformation.

This result is obtained in spite of the fact that the transamination reaction involves both gain and loss of protons in the tautomerization of Schiff base intermediates, the step proposed to be rate-limiting in both the reaction of AAT with its natural substrates (Banks, et al., 1968a; Doonan et al., 1970) and difluoro-oxaloacetate (Briley et al., 1977c). Also because the amino group of pyridoxamine phosphate bound to AAT has been suggested to change its state of ionization in the pH range under study (Morino et al., 1978); the presumably more reactive unionized form (Braunstein, 1973) predominating above ca pH 8.3, a pH variation might be expected.

The observed pH invariance could reflect intramolecular proton transfer, or a shielding of the active site from the environment, or a subtlety in the enzymic transamination reaction at present unidentifiable.

Although studies on the pH variation of the activity of AAT (Jenkins et al., 1959a; Michuda and Martinez-Carrion, 1970) are insufficient to provide information about the effect of pH on the individual interactions of

AAT with its substrates, one set of observations on the effect of pH on enzymic transamination is apparently at variance with the results presented here. The previously-mentioned work of Velick and Vavra, (1962a,b) is alone in providing information for cytoplasmic AAT.

Their work indicated a group on the enzyme (or substrate) whose protonation below ca pH 7.0 directly affected the association of keto-acids with aminic enzyme. Both the Michaelis constant for 2-oxoglutarate (K_a) and the maximal velocity (V_m) decreased with decreasing pH. These kinetic parameters are not directly comparable with the kinetic parameters of the present study as they are more complex in composition and contain macroscopic rate constants pertaining to the other half reaction (ie. the reaction of aspartate with aldimine enzyme). Nevertheless a plot of V_m/K_a versus pH should describe the effect of pH on the association of the keto-acid with aminic-AAT. Such a plot was presented by Velick and Vavra (1962), and indicated the pH variation outlined above. Wada and Morino (1964) have however attributed this observed decrease in K_a with pH, to increasing keto-acid-aldimine-enzyme 'abortive' complex formation with decreasing pH. A similar explanation has been presented to explain in part, the decrease in the rate of exchange transamination between oxaloacetate and aspartate with decreasing pH (Michuda and Martinez-Carrion, 1970)

With the system presented here no such 'abortive' complex formation could inhibit the rate of reaction, as only one 'half turnover' of the enzyme, with initially only aminic enzyme present was studied. Nevertheless care was taken in the choice of difluoro-oxaloacetate concentrations because an excess of this analogue could react with the product aldimine enzyme to form the 'abortive' complex absorbing at 430nm (Briley et al., 1977b), resulting in the rate of reaction measurements at 360nm being underestimates. This phenomenon could possibly account for the greater error on the estimated parameter k_2 observed at low pH. At low pH and high difluoro-oxaloacetate concentration, such an under-estimate of rate would cause a lack of adherence to the scheme of equation (1) and therefore greater divergence of experimental points from best line in plots of computed [ES] versus v (Figs. 11i(b) - 11v(b)). Measurement of the rate of reaction at the 430nm/360nm transition isosbestic point, would have provided an unambiguous measure of aldimine enzyme production.

The above considerations suggest the possibility that at low pH the concentrations of keto-acid required in steady state kinetics to study its interaction with aminic enzyme, are comparable with the levels that would form the 'abortive' complex thus making the two processes indiscernable.

It would also appear that buffer anions have a part to play in the observations of pH variation on the

activity of AAT. Both aldimine and aminic AAT have been shown to bind anions; this binding is competitive with substrate binding and increases with decreasing pH (Cheng et al., 1971; Cheng and Martinez-Carrion, 1972).

The lack of any apparent pH-dependent competitive anion inhibition in the present study can be attributed to the use of low buffer concentrations.

However the possibility also exists that a pH dependent competitive anion inhibitor exactly compensates a decrease in the K_m for difluoro-oxaloacetate with pH. Also Harruff and Jenkins (1978) have indicated the existence of enzyme-substrate-pyrophosphate ternary complexes, which might be a further complication to interpretation. Importantly neither of the above alter the conclusion that the maximum velocity is unaffected by pH.

It would appear that the present finding of a pH insensitivity of the transamination reaction is in agreement with results of Harruff and Jenkins (1978). These authors having obtained similar results on the reaction of erythro-β-hydroxyaspartate with aldimine-AAT concluded that an insensitivity of the substrate affinity and maximum velocity to pH might be a feature of such enzymes "which depend on the formation of multiple enzyme-substrate complexes of comparable stability for their catalytic efficiency".

Results: Part 2

(1) Enzymic Synthesis of L-Difluoro-Aspartic Acid.

L-Difluoro-aspartic acid was prepared by the transamination of difluoro-oxaloacetic acid with L-alanine catalysed by aspartate aminotransferase.

Aldimine-AAT + L-alanine ⟶ Aminic-AAT + pyruvate

Aminic-AAT + difluoro-oxaloacetate ⟶ Aldimine-AAT + difluoro-aspartate

In a typical preparation at 25°C difluoro-oxaloacetate (900mg) and alanine (9.2g) in water (100ml, brought to pH 7.4 with 5M-NaOH)were introduced at a rate of 20ml/day into a stirred solution of AAT (457mg, 328,000 e.units, 9.4 μMoles). The enzyme solution (200ml) contained 1mM-dithiothreitol and 0.02% $^{w}/_{v}$-sodium azide and was brought to pH 7.4 with M/50-NaOH.

The progress of the reaction was followed by measuring pyruvate product. In an aliquot of the reaction mixture pyruvate production was measured spectrophotometrically by following the decrease in the optical density at 340nm caused by the oxidation of NADH in the presence of lactate dehydrogenase. The system (1ml, in a 1cm semi-micro quartz cuvette) comprised 0.2mM-NADH and lactate dehydrogenase (5 μg) in 100mM-pyrophosphate buffer, pH 7.4. Over the period of the reaction (5 days) enzyme activity, monitored by standard assay, was also followed. The slow increase in acidity (ca. 0.5 pH units/day) in this unbuffered reaction mixture was corrected by regular additions of M/50-NaOH.

After 5 days enzyme was separated by dialysis against partial vacuum. The presence of difluoro-aspartate in the resulting diffusate was checked by ^{19}F n.m.r. (Fig. 13) prior to freeze-drying.

The results of monitoring this preparation of difluoro-aspartic acid are presented in Figure 12. It was found that a similarly efficient preparation of difluoro-aspartate could be effected by using a crude enzyme preparation (Step 3 of AAT preparation, Table 1i) however in these preparations it was not found possible to measure any pyruvate product.

Difluoro-aspartic acid was purified by chromatography on AG1-X8 anion exchange resin (Bio-Rad; 200-400mesh, formate form). The sample (9.2g) dissolved in 3M-formic acid (60ml) was applied to a column (26cm x 1.8cm) of this resin and the column was eluted with 3M-formic acid (ca 300ml) until the eluant was no longer ninhydrin-positive. Difluoro-aspartic acid was then eluted with 2M-ammonium formate, pH 3.7, fractions (10ml) were collected and tested for the presence of difluoro-aspartic acid by using ^{19}F n.m.r.. Difluoro-aspartate was found to be eluted with the change in eluant pH from ca 2 to 3.5, caused by the change in eluting medium.

The difluoro-aspartate-containing fractions (70ml) were collected and freeze-dried. The resulting residue

Figure 12

Enzymic preparation of L-difluoro-aspartic acid.

A solution (100ml) of difluoro-oxaloacetate (900mg) and L-alanine (9.2g) pH 7.4 was introduced (continuous line) at the rate of 20ml/day into a stirred solution of AAT (25°C; pH 7.4). The rate of addition is expressed in terms of the rate of difluoro-oxaloacetate addition. Enzyme activity (●) was monitored and pyruvate assayed (▲). Pyruvate production is expressed in terms of the corresponding consumption of difluoro-oxaloacetate (mg), (details in text).

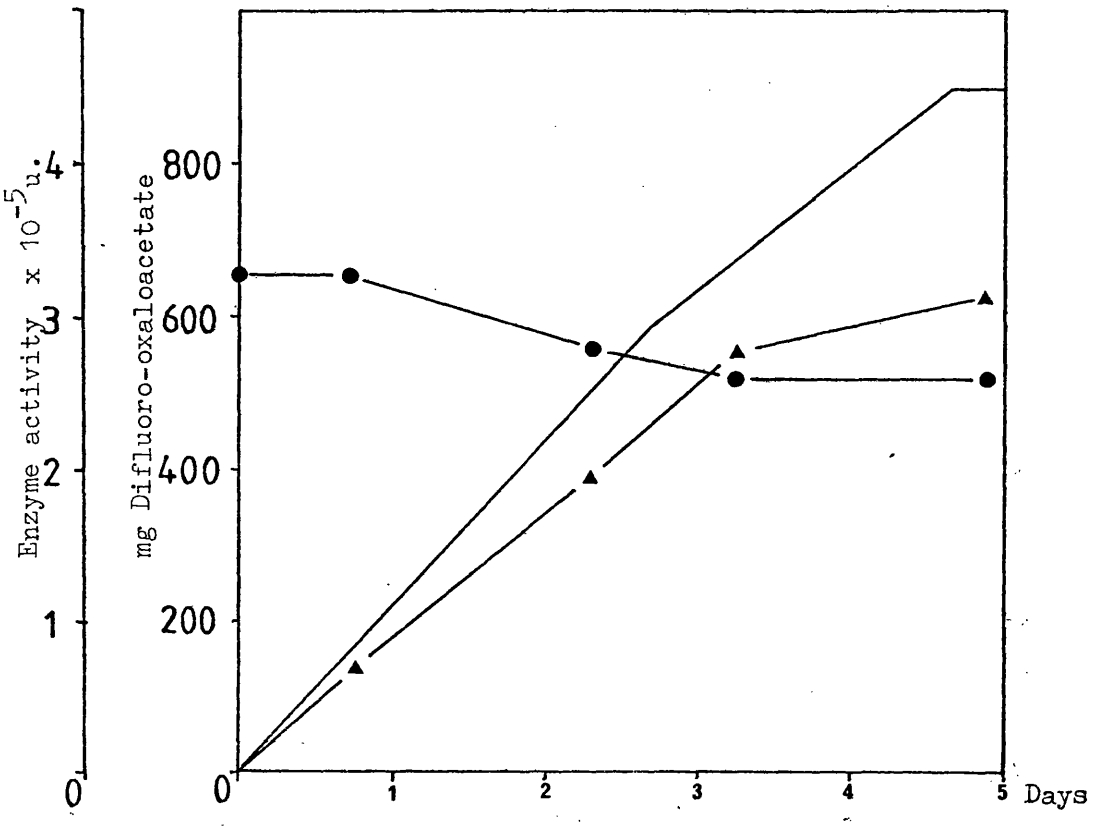

Enzyme activity x 10^{-5} u.
0
1
2
3
4
mg Difluoro-oxaloacetate
0
200
400
600
800
0
1
2
3
4
5 Days

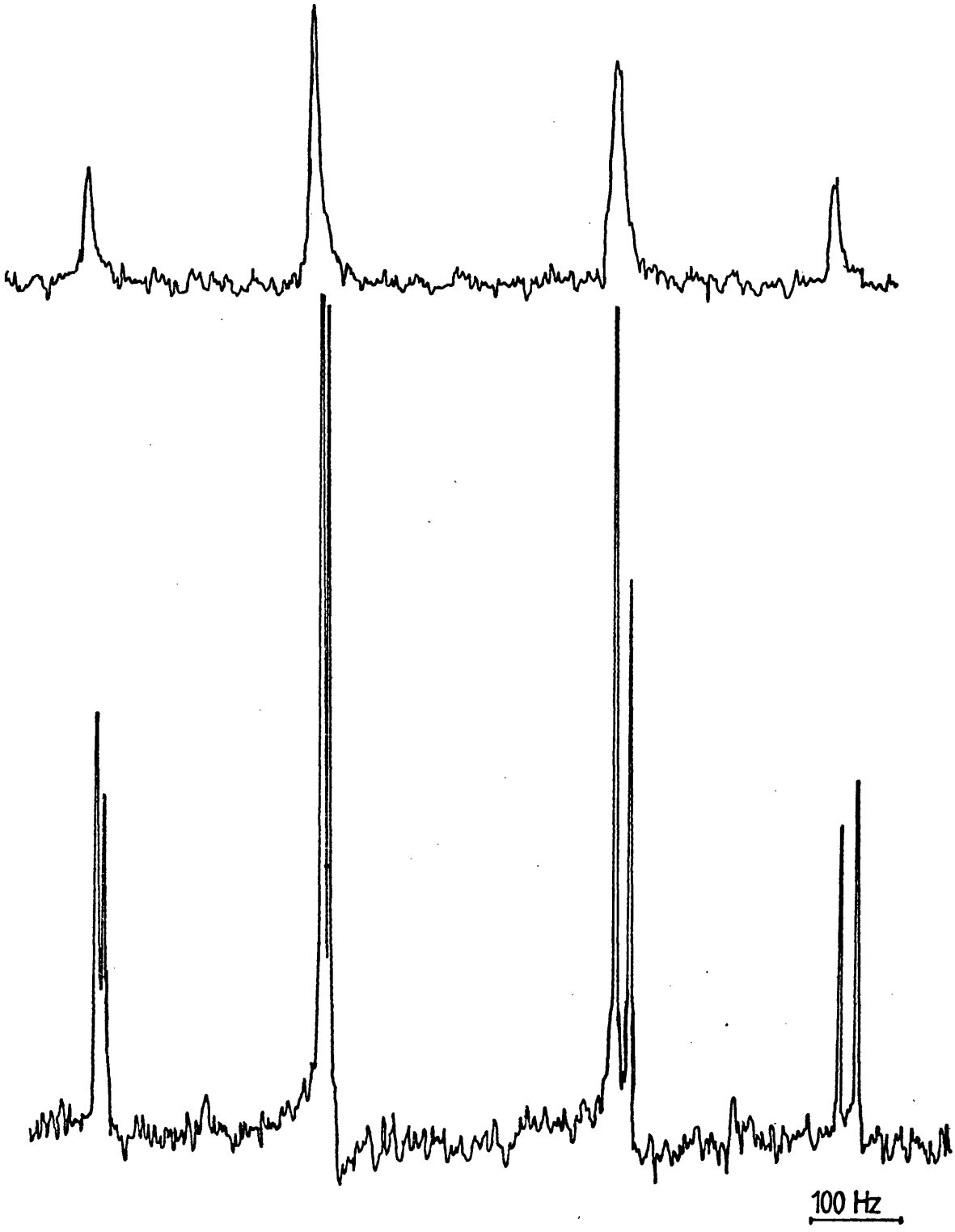
Figure 13

^{19}F n.m.r. spectrum of L-difluoro-aspartic acid.

The nuclear magnetic resonance spectrum of purified L-difluoro-aspartic acid (30mg in D_2O, 1ml , pH 4.7) was recorded at 94 MH_z on a JEOL PS 100 n.m.r. spectrometer in a 5mm diameter tube with a coaxial capillary containing trifluoro-acetic acid as an internal lock signal. The signal due to difluoro-aspartate, centred 3020 H_z upfield from trifluoro-acetic acid, has the following n.m.r. parameters:-

J_{AX}	$7.3H_z$
J_{BX}	$18.3H_z$
J_{AB}	$280H_z$
$\upsilon_A - \upsilon_B$	$596H_z$

Superimposed is the proton decoupled spectrum.


100 Hz

was triturated with 95% ethanol and removed by filtration. Further purification was afforded by dissolving the solid in the minimum volume of distilled water (ca 1ml) and reprecipitating with a large excess of 95% ethanol to give the product as a white crystalline powder (532mg), mp $> 126^{\circ}C$ decomposes.

Elemental analysis was consistent with that of the mono-ammonium salt of difluoro-aspartic acid monhydrate.

	$C_4H_{10}N_2F_2O_5$			
Calculated	C23.51	H4.90	N13.71	F18.61
Found	C23.64	H4.86	N13.65	F18.62
	C23.50	H4.89	N13.77	F18.42

Titration with base (Fig. 14) gave a molecular weight of 202, confirming that the acid was present as its mono-ammonium salt. (M.Wt. of $^{-}OOCCF_2CH(NH_3)^{+}COO^{-}NH_4^{+}$ (H_2O) = 204)

The second pK_a of the product was found to be 9.31 (Fig. 14) and upon titration of this ammonia was detected. The pK_a of the amino group of difluoro-aspartate was found to be 7.18. The difluoro-aspartate was further characterised by measurements of its optical activity. (Table 3) which showed the compound to be laevorotatory (cf.L-aspartate $[\alpha]_D^{24}$ + 24.6; 6N HCl , conc. = 2.002 g/100ml).

Mono-ammonium-L-difluoro-aspartic acid monhydrate (hereafter termed difluoro-aspartate) solid was stored under vacuum over P_2O_5 at $-17^{\circ}C$; under these conditions it was found to be stable for periods in excess of two

Table 3

Optical rotatory dispersion data for L-difluoro-aspartic acid.

Specific rotation ($[\alpha]$) calculated at various wavelengths from observation of the optical rotation (α) exhibited by a solution (103mg/11ml) of the mono-ammonium salt of difluoro-aspartic acid monohydrate in 1M HCl (1dm.cell; Perkin Elmer 141 Polarimeter).

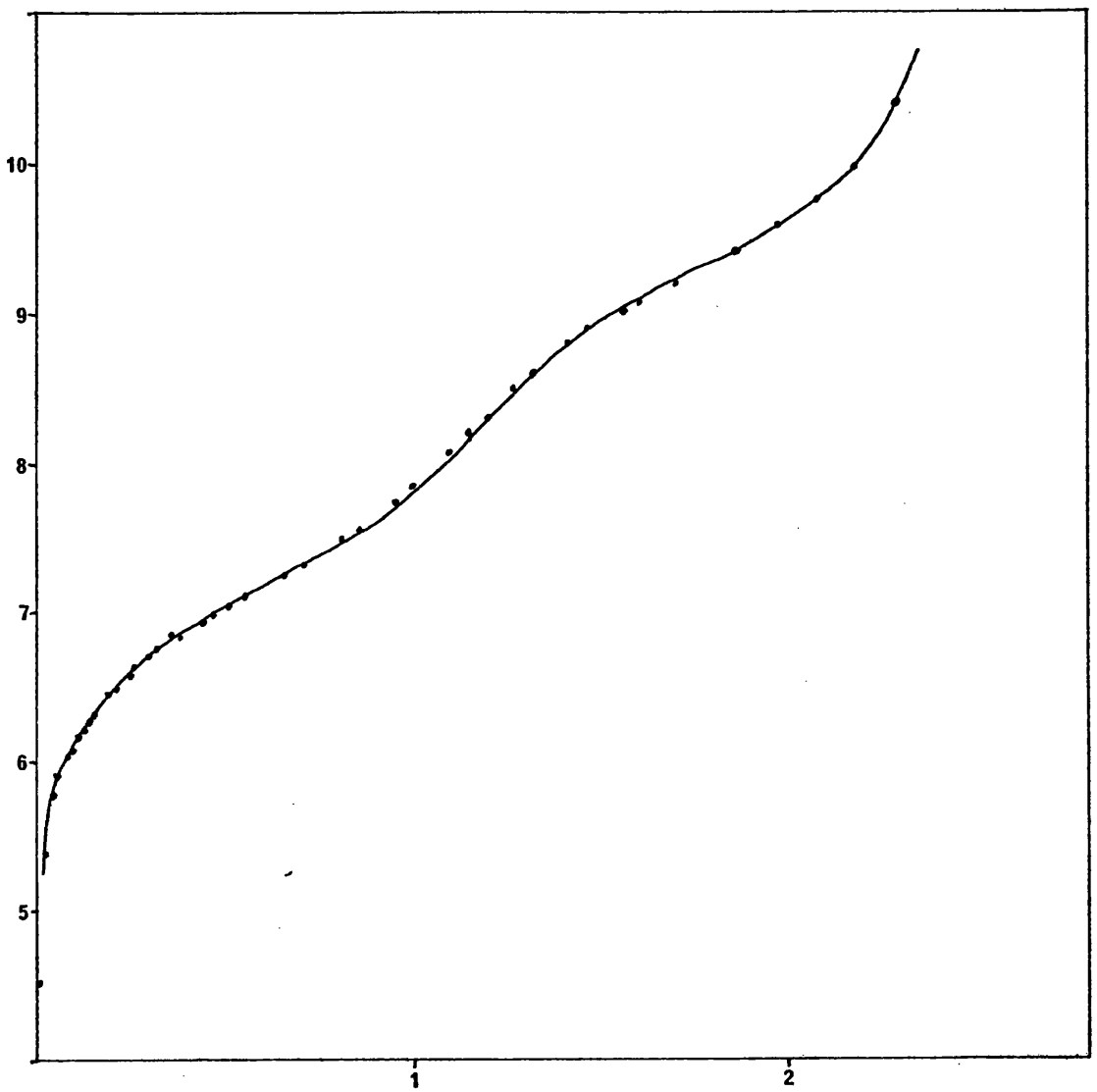
λ_{nm}	α^{o}	$[\alpha]$
589	-0.030	-3.204
578	-0.034	-3.631
546	-0.043	-4.592
436	-0.070	-7.476
365	-0.137	-14.631

Figure 14

Characterisation of difluoro-aspartic acid by titration with base.

A solution of difluoro-aspartic acid (mono-ammonium salt, monohydrate; 21.3mg/1.1ml) prepared as described was titrated with standard sodium hydroxide (0.0896M) by using a Radiometer (Copenhagen) titration assembly TTA 31 coupled to the pH meter 26. Experimental points (•) pH experimental corrected for standard buffers and ionic strength using the mean activity coefficient of HCl (ionic strength 0.124); line drawn from the parameters $pK_1 = 7.18$; $pK_2 = 9.31$; molecular weight determination gave 202 (calculated 204), determined from the data by the method of Grzybowski (personal communication).

The method of Grzybowski is a computation procedure centering on the curve fitting method of Schwarzenbach and also includes subroutines for preparation of data and statistical analysis.


pH
10
9
8
7
6
5
1
2
Vol. of titrant (ml)

months, as judged by elemental analysis. Stock solutions of difluoro-aspartate made up in the appropriate buffer were stored at -17°C for not more than two weeks.

(2) Attempted Chemical Synthesis of D,L-Difluoro-Aspartic Acid.

The chemical synthesis of D,L-difluoro-aspartic acid was attempted by using a variety of procedures. The methods employed will only be outlined briefly here as none of the schemes proved to be totally satisfactory.

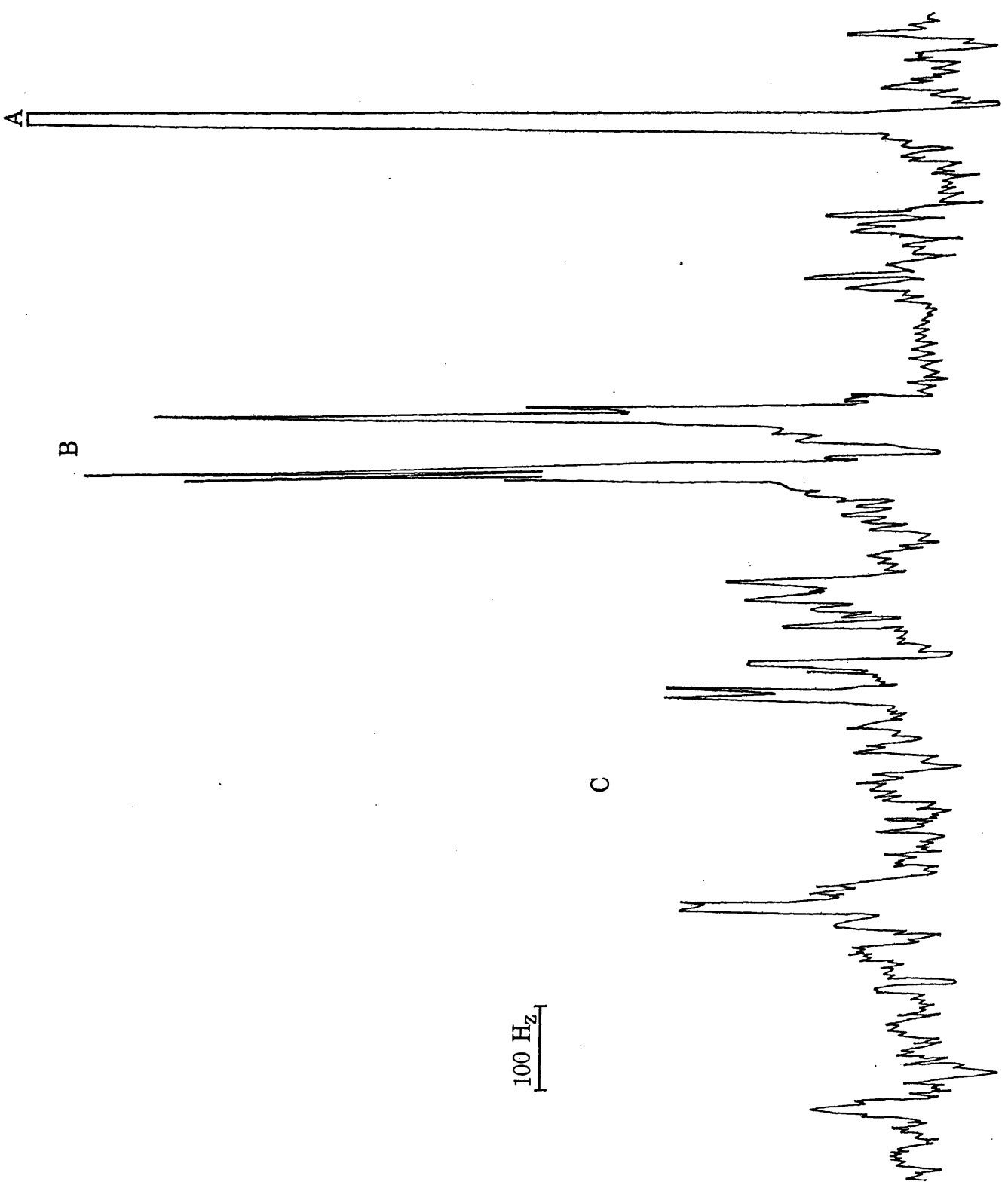
(2) i). Reductive-amination of difluoro-oxaloacetic acid.

The method used was that of Borch et al., (1971) for the synthesis of α-amino acids from their parent keto acids. Difluoro-oxaloacetic acid (186mg) and ammonium bromide (500mg) in dry methanol (30ml) was adjusted to pH 7.0 by addition of solid K_2CO_3 and using a pH meter (a trace of water was required to dissolve the potassium salt of difluoro-oxaloacetic acid so produced). After 30 m sodium cyanohydridoborate (125mg) was added and the whole was left stirring for 48h. The product of this reaction were assesed by ^{19}F n.m.r. and found tc be variable: Either difluoro-oxaloacetate failed to react or difluoro-malate (with a trace of difluoro-aspartate) was formed. An example of the ^{19}F n.m.r. spectrum of the products of one reaction is given in Figure 15. The presence of difluoro-malate, difluoro-oxaloacetic and difluoro-aspartate were based on their known ^{19}F n.m.r. spectra (Smith, 1978), (Fig. 13). When the reaction was carried out at pH 6.5 difluoro-malate was the primary product whereas at pH 8.0 unreacted difluoro-oxaloacetate predominated. Replacement of

Figure 15

^{19}F n.m.r. spectrum of the products of an attempt to reductively aminate difluoro-oxaloactic acid.

The spectrum of the products of an attempt to reductively aminate difluoro-oxaloacetic acid was recorded (in D_2O; pH 7.0) as described in Figure 13 and illustrates the spectra arising from unreacted difluoro-oxaloacetate (A), difluoro-malate (B) and difluoro-aspartate (C), (Smith, 1978).


A
B
C
100 Hz

methanol as solvent by various combinations of dioxan/methanol produced similar results.

(2) ii). Reductive amination of the diethyl ester of difluoro-oxaloacetate.

a. Reduction with hydrogen and palladium black.

A solution of diethyl-difluoro-oxaloacetate (1g) in dry methanol (60ml) saturated with ammonium bromide was hydrogenated at 10 Atmospheres in the presence of palladium black (1.5g; 10% on charcoal) for 24h. The final reaction mixture was found to contain the unreacted ester as judged by I.R. spectroscopy.

b. Reduction with sodium cyanohydridoborate.

Identical conditions as described in (a) were employed except that sodium cyanohydridoborate (0.6g) was used as the reductant. After 24h the methanol was removed under vacuum and the product was extracted with dry ether (3 x 200ml). Evaporation yielded an amber oil (1.2g). This product although contaminated with cyanohydridoborate (presumably as the cyanoborane adduct, Weidig et al.,1974) exhibited a proton n.m.r. spectrum attributable to that of the diethyl ester of difluoro-aspartate (Fig. 16).

Hydrolysis of the product obtained as above without further purification was attempted as follows:-

Figure 16

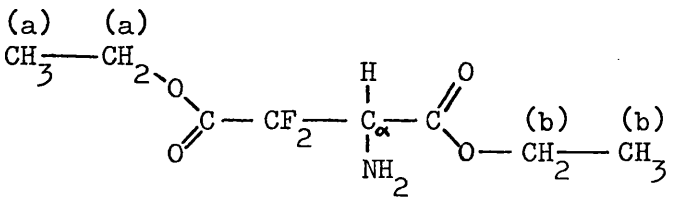
Proton n.m.r. spectrum of the product of the reductive amination of diethyl-difluoro-oxaloacetate.

The proton n.m.r. spectrum of the product (30mg in deuteroacetone) was obtained with a JEOL PS 100 n.m.r. spectrometer with tetramethylsilane as an internal standard. The position of the amino group was confirmed by observing the disappearance of its signal in D_2O (A) and the α-proton by ^{19}F decoupling (B). The relative area under each peak was measured with the intergral curve shown.

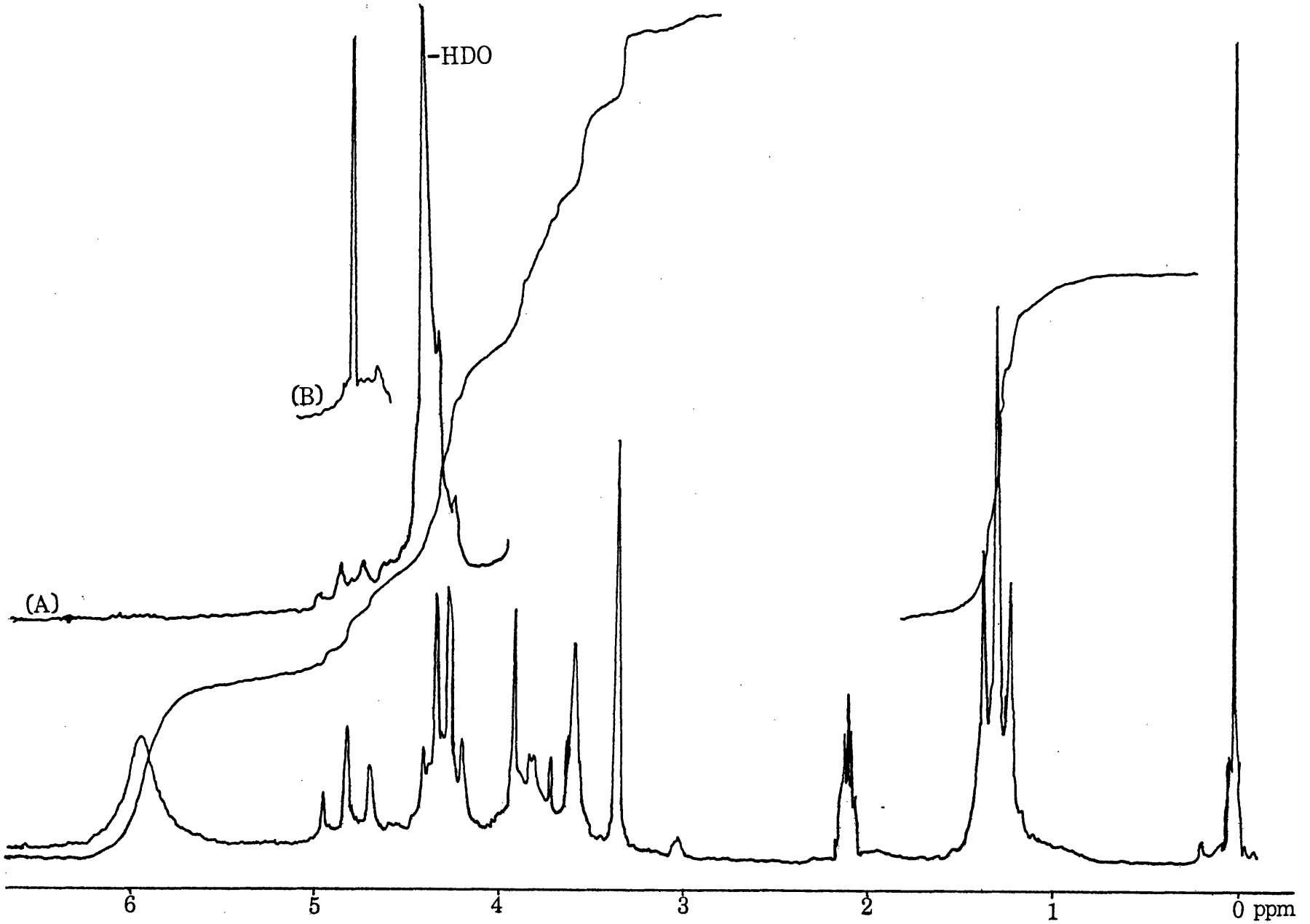
The spectrum was assigned to the diethyl ester of difluoro-aspartate as follows:-

Group[1]	Chemical shift (σ) [2]
NH_2	5.95 (broad singlet) lost in D_2O (A)
α-proton	4.82 (triplet) singlet on ^{19}F decoupling (
$CH_{2(a)}$	4.30 (quartet)
$CH_{2(b)}$	3.80 (quartet)
$CH_{3(a+b)}$	1.30 (triplet)
Extraneous peaks	3.40 (singlet)
	3.60 (singlet)

(1)



(2) For nomenclature and interpretation see Jackman (1959)


-HDO
(B)
(A)
6
5
4
3
2
1
0 ppm

1. Refluxing in 33% HCl for 1½h.
2. Incubation in 3M-formic acid at room temperature for 4 days.
3. Incubation in 5%-KOH in 95% ethanol in the cold for 4 days.
4. Incubation with a buffered solution of the enzyme esterase.

However none of these schemes produced free difluoro-aspartate acid as judged by the criterion of its characteristic ^{19}F n.m.r. spectrum.

(2).iii). Reaction of diethyl sodium phthalimidomalonate with ethyl chloro-difluoro-acetate.

The method is based on the procedure of Dunn and Smart (1930) for the synthesis of D,L-aspartate from ethyl chloro-acetate. Diethyl sodium phthalimidomalonate was prepared by the method of Barger and Weichselbaum (1943) and refluxed for 30h. with ethyl chloro-difluoro-acetate, prepared from chloro-difluoro-acetic acid by standard procedure. Volhard analysis (for Cl^-) of the reaction mixture however indicated no reaction.

(3) Inhibition Kinetic Study of the Interaction of Difluoro-Aspartate with Aspartate Aminotransferase.

(3) i). Preliminary experiments indicated that difluoro-aspartate was capable of inhibiting the AAT-catalysed transamination between 2-oxoglutarate and aspartate. The interaction of difluoro-aspartate with AAT was therefore studied by determining its mode of inhibition of the enzyme under steady state conditions.

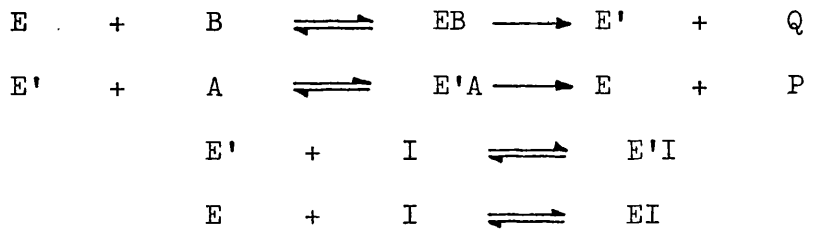
A scheme is presented in Figure 17 for the possible interactions of difluoro-aspartate with AAT. This is based both on the known substrate kinetics of this enzyme, and on the observations that most dicarboxylic acid substrate analogues can bind to both aldimine and aminic AAT (Haarhoff, 1969). The rate equation corresponding to this scheme:-

$$v = \frac{V\,[A]\,[B]}{[A]\,[B] + [B]\,K_a(1+[I]/K_i') + [A]K_b(1+[I]/K_i)} \quad \ldots(1)$$

where K_a and K_b are the Michaelis constants for 2-oxoglutarate and aspartate respectively, K_i' and K_i are the dissociation constants of the aminic transaminase-inhibitor ('abortive') and the aldimine enzyme-inhibitor complexes respectively, A, B, and I are defined in Figure 17.

Figure 17

Scheme for the possible interaction of difluoro-aspartate with aspartate aminotransferase.



E', aminic-aspartate aminotransferase; E, aldimine-enzyme;

A, oxo-acid substrate (2-oxoglutarate);

B, amino-acid substrate (aspartate);

Q, oxo-acid product (oxaloacetate);

P, amino-acid product (glutamate);

I, inhibitor (difluoro-aspartate).

Equation (1) may be written:-

$$v = \frac{\dfrac{V\,[B]}{[B] + K_b\,(1+[I]/K_i)}\,[A]}{\dfrac{K_a\,(1 + [I]/K_i')\,[B]}{K_b\,(1 + [I]/K_i) + [B]} + [A]} \qquad \ldots(2)$$

or

$$v = \frac{\dfrac{V\,[A]}{[A] + K_a(1 + [I]/K_i')}\,[B]}{\dfrac{K_b(1 + [I]/K_i)\,[A]}{K_a(1 + [I]/K_i') + [A]} + [B]} \qquad \ldots(3)$$

Equation (2) is of the form

$$v = \frac{V_a^{app}\,[A]}{K_a^{app} + [A]} \qquad \ldots(4)$$

where

$$V_a^{app} = \frac{V\,[B]}{[B] + K_b\,(1 + [I]/K_i)} \qquad \ldots(5)$$

$$K_a^{app} = \frac{K_a(1 + I/K_i')\,[B]}{K_b(1 + I/K_i) + [B]} \qquad \ldots(6)$$

Equation (3) is of the form

$$v = \frac{V_b^{app}\,[B]}{K_b^{app} + [B]} \qquad \ldots(7)$$

where

$$V_b^{app} = \frac{V\,[A]}{[A] + K_a(1 + [I]/K_i')} \qquad \ldots(8)$$

$$K_b^{app} = \frac{K_b(1 + [I]/K_i)\,[A]}{K_a(1 + [I]/K_i') + [A]} \qquad \ldots(9)$$

Equations (2) and (3) predict mixed inhibition, therefore the most straightforward method of analysis was to investigate the effect of difluoro-aspartate (I) on the parameters $1/V_a^{app}$, $1/V_b^{app}$, K_a^{app}/V_a^{app} and K_b^{app}/V_b^{app}

The malate dehydrogenase coupled assay of Karmen (1955) was used to study the effect of difluoro-aspartate on the initial velocity (v) of the AAT-catalysed transamination between 2-oxoglutarate and aspartate. In a 1cm pathlength semi-micro quartz cuvette the system (1ml) at 25°C comprised 100mM-pyrophosphate buffer, pH 7.4, 0.2mM-NADH, malate dehydrogenase (55 i.u.) and four systematically-varied concentrations of 2-oxoglutarate [A], aspartate [B] and difluoro-aspartate [I]. The initial velocity was measured after the addition of AAT to a final concentration of 0.55nM (sites) and is expressed in turnover units. Values of the parameters

K_a^{app}/V_a^{app} and $1/V_a^{app}$ were obtained from direct linear plots (Cornish Bowden and Eisenthal, 1978) of initial rate (v) against concentrations of 2-oxoglutarate [A] at different fixed concentrations of aspartate [B] for each difluoro-aspartate [I] concentration. Values of K_b^{app}/V_b^{app} and $1/V_b^{app}$ were obtained from converse plots.

Primary kinetic data are presented in Figures 18, 19, 20 and 21 as plots of [A] /v versus [A] and in Figures 23, 24, 25 and 26 as plots of [B] / v versus [B] . For these plots rearrangement of equation (2) and (3) respectively gives:-

$$\frac{[A]}{v} = \frac{K_a(1 + [I]/K_i')}{V} + \frac{\frac{1}{V}\,[B]}{[B] + K_b(1 + [I]/K_i)}\,[A] \qquad \ldots(10a)$$

$$\frac{[A]}{v} = \frac{K_a^{app}}{V_a^{app}} + \frac{1}{V_a^{app}}[A] \qquad \ldots(10b)$$

$$\frac{[B]}{v} = \frac{K_b(1 + [I]/K_i)}{V} + \frac{\frac{1}{V}\,[A]}{[A] + K_a(1 + [I]/K_i')}\,[B] \qquad \ldots(11a)$$

$$\frac{[B]}{v} = \frac{K_b^{app}}{V_b^{app}} + \frac{1}{V_b^{app}}[B] \qquad \ldots(11b)$$

Equations (10) and (11) predict for a fixed concentration of difluoro-aspartate [I] with the various concentrations of the co-substrate a family of straight lines having

a common intercept on the ordinate and varying slope. This was found to be the case and is characteristic of the substituted enzyme mechanism.

(3) ii). The interaction of difluoro-aspartate with aminic form of AAT.

The forms of the enzyme with which difluoro-aspartate interacts are kinetically distinguishable. The dissociation constant of the aminic enzyme-difluoro-aspartate ('abortive') complex (K_i') may be determined as the competitive element of the inhibition with respect to the substrate that interacts with this form of the enzyme ie. 2-oxoglutarate. Under the scheme of Figure 17 the parameters K_a^{app}/ V_a^{app} is sensitive only to this element of the inhibition. Consideration of equations (5) and (6) yields the following expression for this parameter:-

$$\frac{K_a^{app}}{V_a^{app}} = \frac{K_a (1 + [I]/K_i')}{V} \qquad \ldots(12)$$

From which it can be seen that K_a^{app} / V_a^{app} is independent of the co-substrate concentration and dependant only on difluoro-aspartate concentration, as was found.

Thus four estimates of K_a^{app} / V_a^{app} exist for each co-substrate concentration. A plot of mean K_a^{app} / V_a^{app} versus difluoro-aspartate concentration [I] (Fig. 22) was linear and indicated a value of 44mM for K_i'. Some

Figure 18

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [A]/v versus [A] for the various aspartate [B] concentrations; (o) 4.0mM, (●) 2.0mM, (△) 1.0mM, (▲) 0.5mM, and no difluoro-aspartate. The lines were drawn from the parameters K_a^{app}/V_a^{app} and $1/V_a^{app}$ determined from the data as described in text.

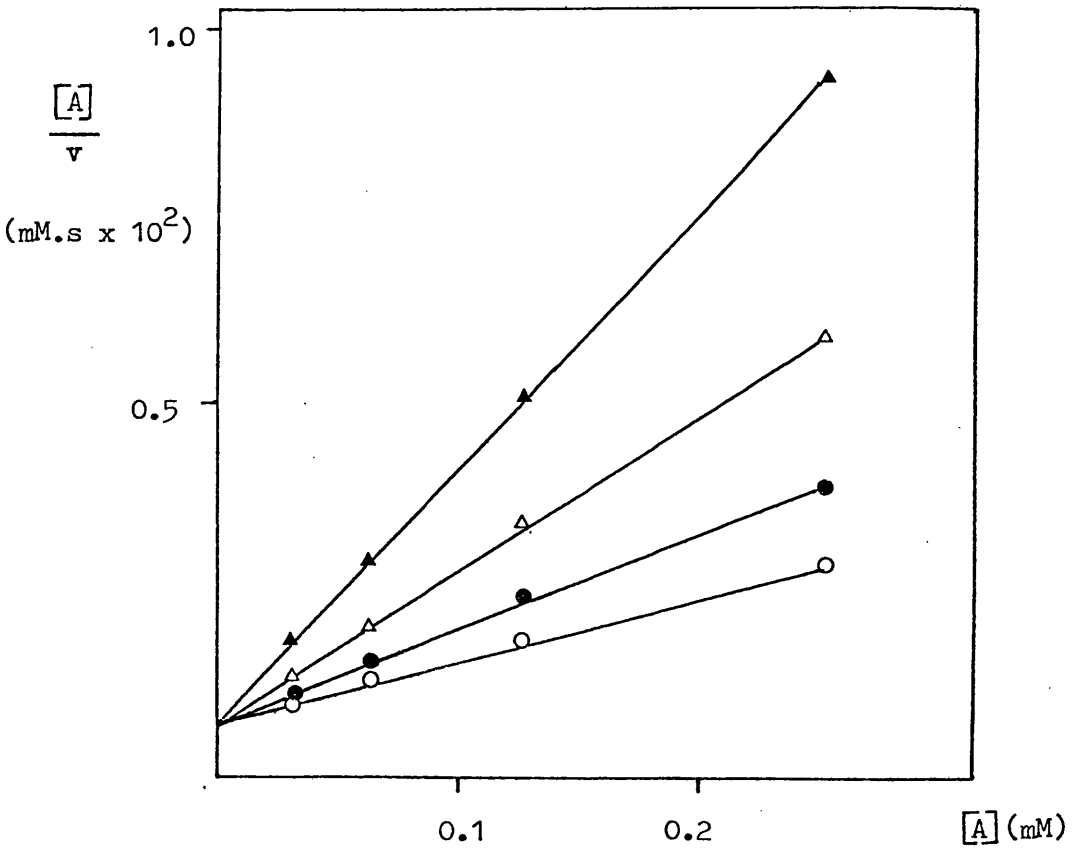

1.0
[A]
v
(mM.s x 10²)
0.5
0.1
0.2
[A] (mM)

Figure 19

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [A]/v versus [A] for the various aspartate [B] concentrations; (○) 4·0mM , (●) 2·0mM , (△) 1·0mM , (▲) 0·5mM , with the addition of difluoro-aspartate to a final concentration of 3·75mM. The lines were drawn from the parameters K_a^{app} / V_a^{app} and $1/V_a^{app}$ determined from the data as described in text.

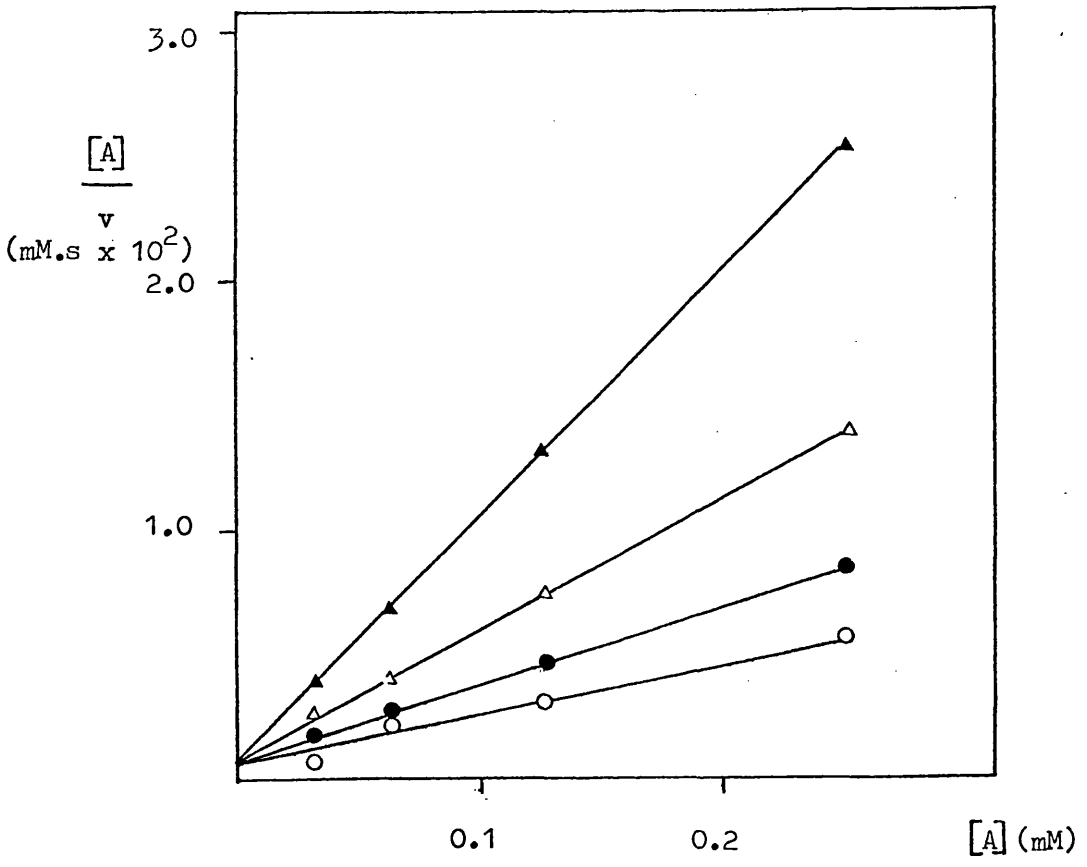

3.0
2.0
1.0
[A]
v
(mM.s x 10²)
0.1
0.2
[A] (mM)

Figure 20

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [A]/v versus [A] for the various aspartate [B] concentrations; (○) 4.0mM, (●) 2.0mM, (△) 1.0mM, (▲) 0.5mM, with the addition of difluoro-aspartate to a final concentration of 7.5mM. The lines were drawn from the parameters K_a^{app}/ V_a^{app} and 1/ V_a^{app} determined from the data described in the text.

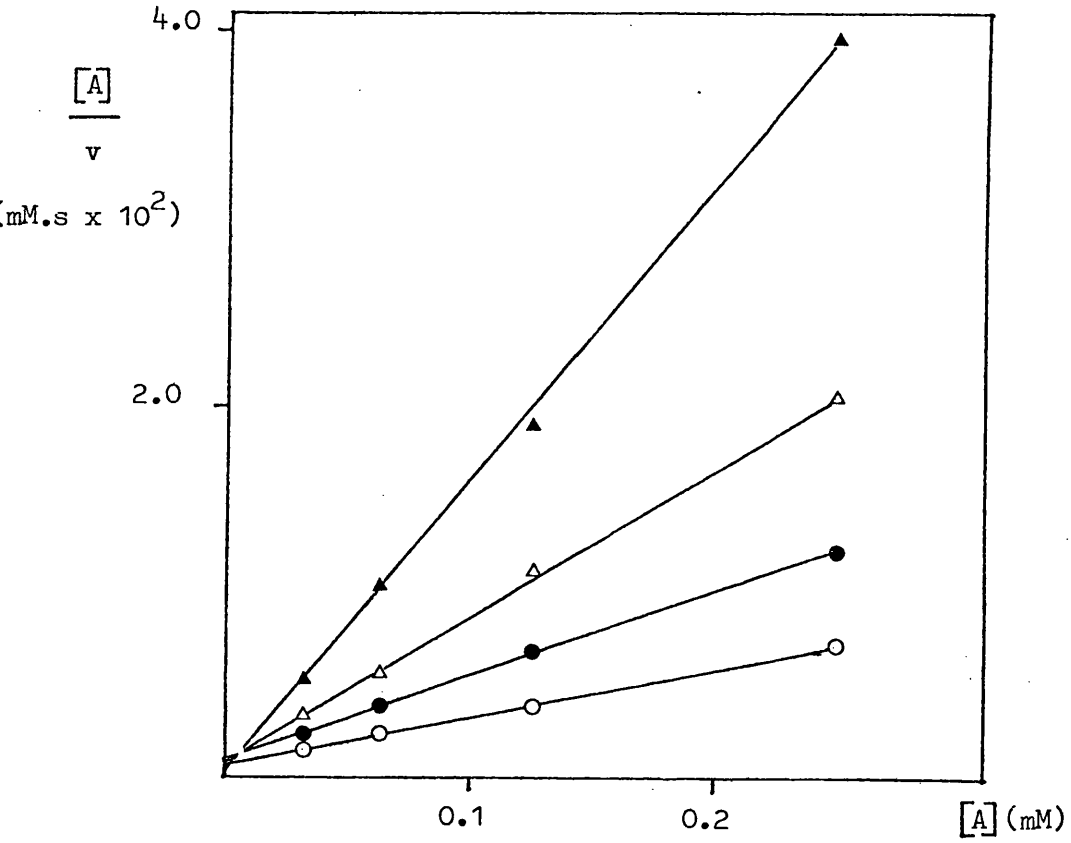

4.0
[A]
v
(mM.s x 10²)
2.0
0.1
0.2
[A] (mM)

Figure 21

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [A]/v versus [A] for the various aspartate [B] concentrations; (○) 4.0mM, (●) 2.0mM, (△) 1.0mM, (▲) 0.5mM, with the addition of difluoro-aspartate to a final concentration of 15mM. the lines were drawn from the parameters K_a^{app}/ V_a^{app} and 1/ V_a^{app} determined from the data as described in the text.

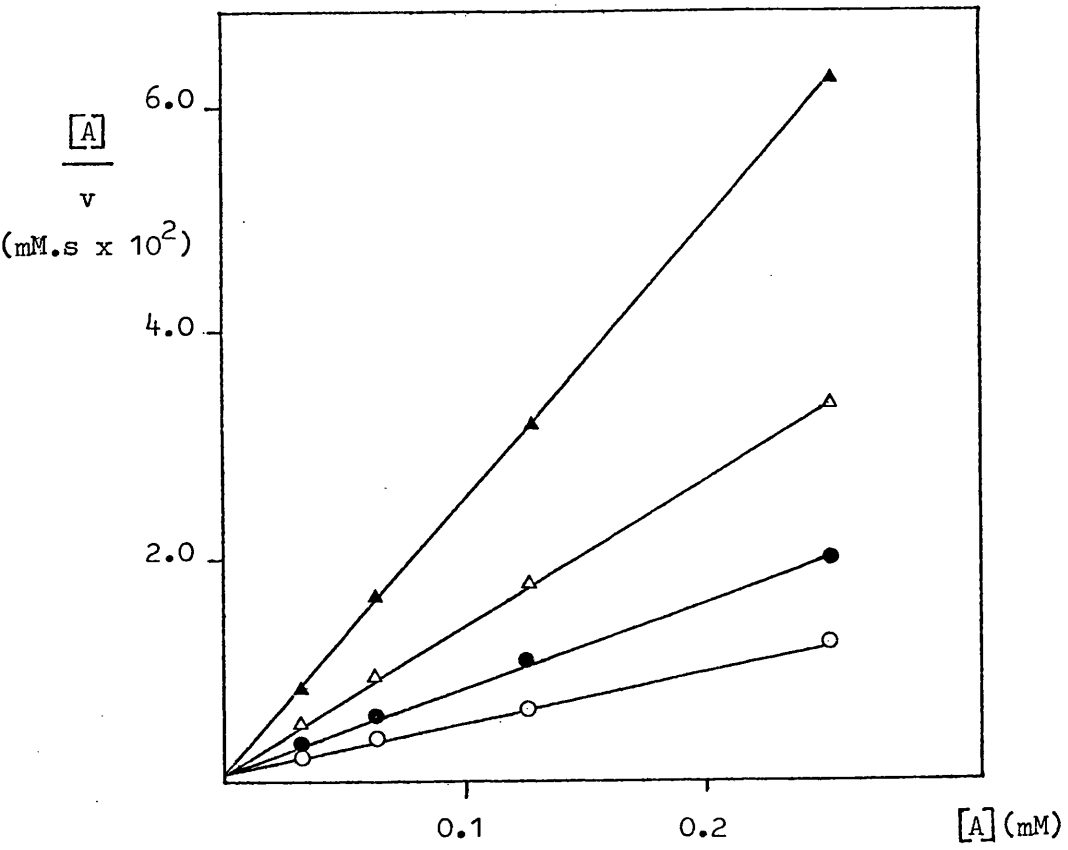

[A]
v
(mM.s x 10²)
6.0
4.0
2.0
0.1
0.2
[A] (mM)

Figure 22

Secondary plot of the effect of difluoro-aspartate concentration [I] on K_a^{app} / V_a^{app}.

Data points (■) are the mean estimates of K_a^{app} / V_a^{app} obtained from studies of the variation of initial rate (v) with respect to 2-oxoglutarate [A] concentration at the various aspartate [B] concentrations and at constant difluoro-aspartate. The line was drawn by linear regression analysis.

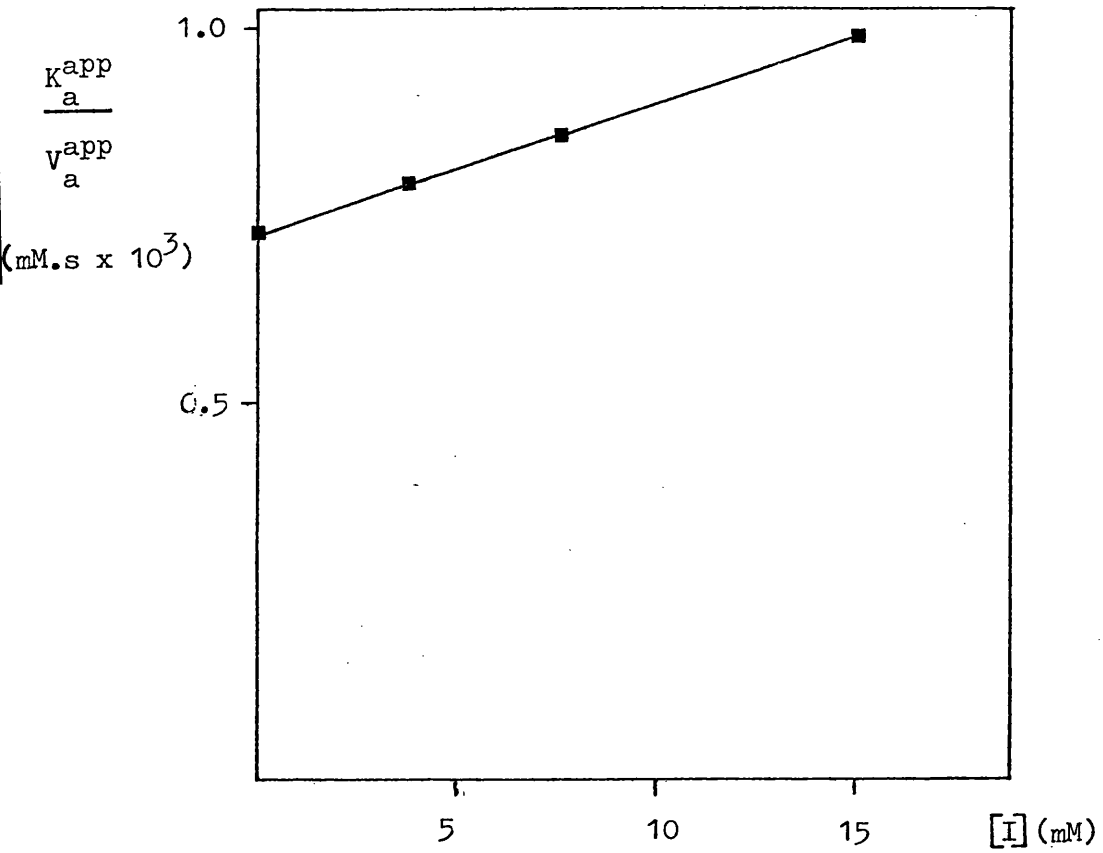

1.0
0.5
$\frac{K_a^{app}}{V_a^{app}}$
(mM.s x 10^3)
5
10
15
[I] (mM)

Figure 23

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [B]/v versus [B] for the various 2-oxoglutarate [A] concentrations; (○) 0.25mM , (●) 0.125mM , (△) 0.063mM , (▲) 0.031mM, and no difluoro-aspartate. The lines were drawn from the parameters K_b^{app} / V_b^{app} and 1/ V_b^{app} determined from the data as described in the text.

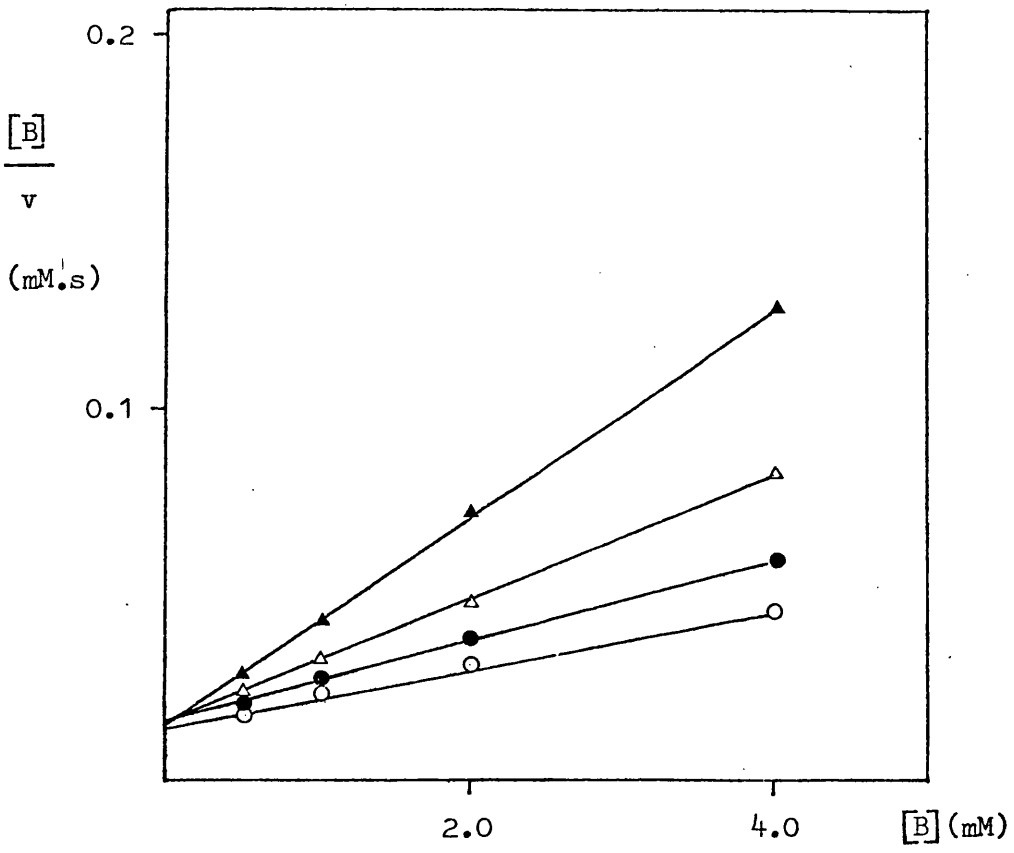

0.2
[B]
v
(mM.s)
0.1
2.0
4.0
[B] (mM)

Figure 24

Primary kinetic data for the inihibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [B]/v versus [B] for the various 2-oxoglutarate [A] concentrations; (○) 0.25mM, (●) 0.125mM, (△) 0.063mM, (▲) 0.031mM, with the addition of difluoro-aspartate to a final concentration of 3.75mM. The lines were drawn from the parameters K_b^{app}/ V_b^{app} and 1/ V_b^{app} determined from the data as described in the text.

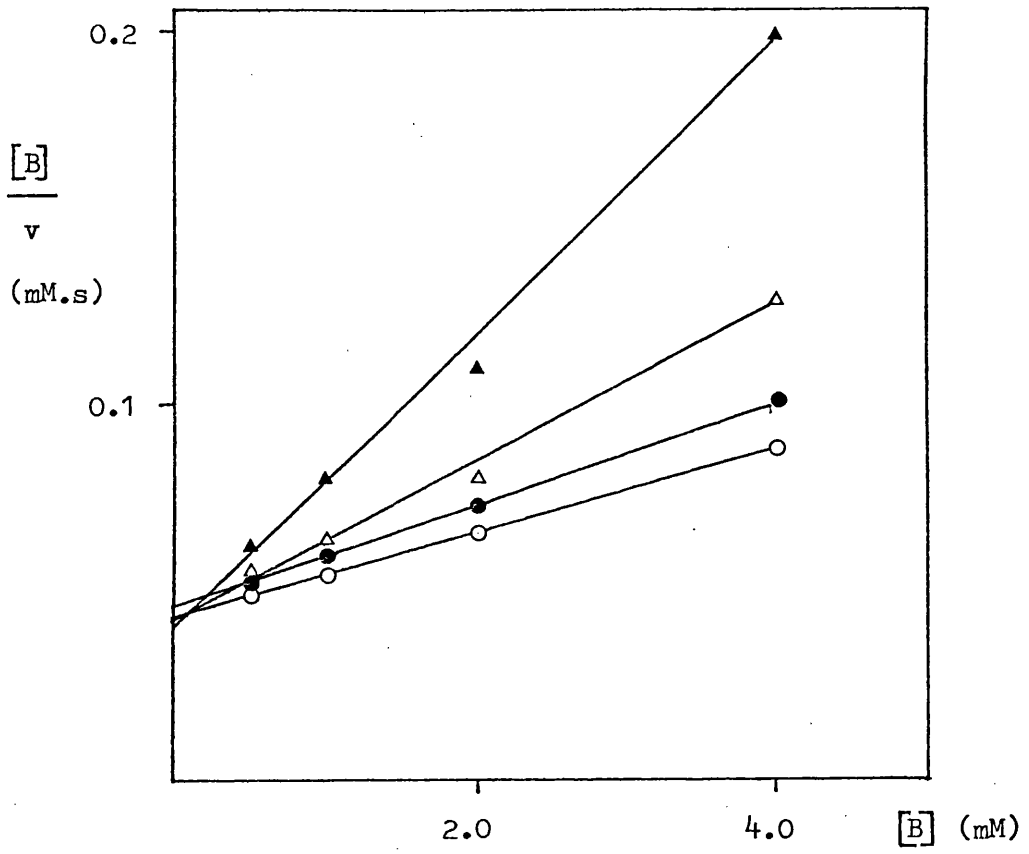

0.2
0.1
[B]
v
(mM.s)
2.0
4.0
[B] (mM)

Figure 25

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [B]/v versus [B] for the various 2-oxoglutarate [A] concentrations ; (○) 0.25mM, (●) 0.125mM, (△) 0.063mM, (▲) 0.031mM, with the addition of difluoro-aspartate to a final concentration of 7.5mM. The lines were drawn from the parameters K_b^{app}/ V_b^{app} and $1/ V_b^{app}$ determined from the data as described in the text.

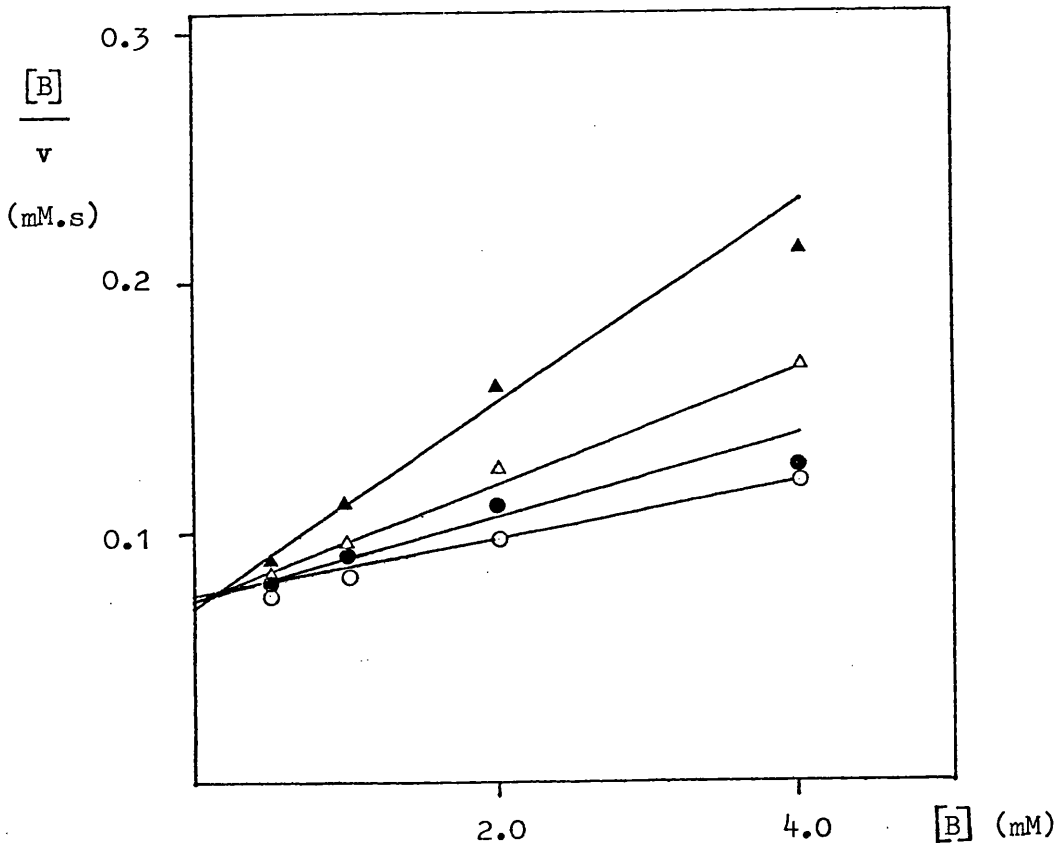

0.3
[B]
v
(mM.s)
0.2
0.1
2.0
4.0
[B] (mM)

Figure 26

Primary kinetic data for the inhibition of aspartate aminotransferase by difluoro-aspartate.

Plots of [B]/v versus [B] for the various 2-oxoglutarate [A] concentrations ; (○) 0.25mM, (●) 0.125mM, (△) 0.063mM, (▲) 0.031mM, with the addition of difluoro-aspartate to a final concentration of 15mM. The lines were drawn from the parameters K_b^{app}/ V_b^{app} and 1/ V_b^{app} determined from the data as described in the text.

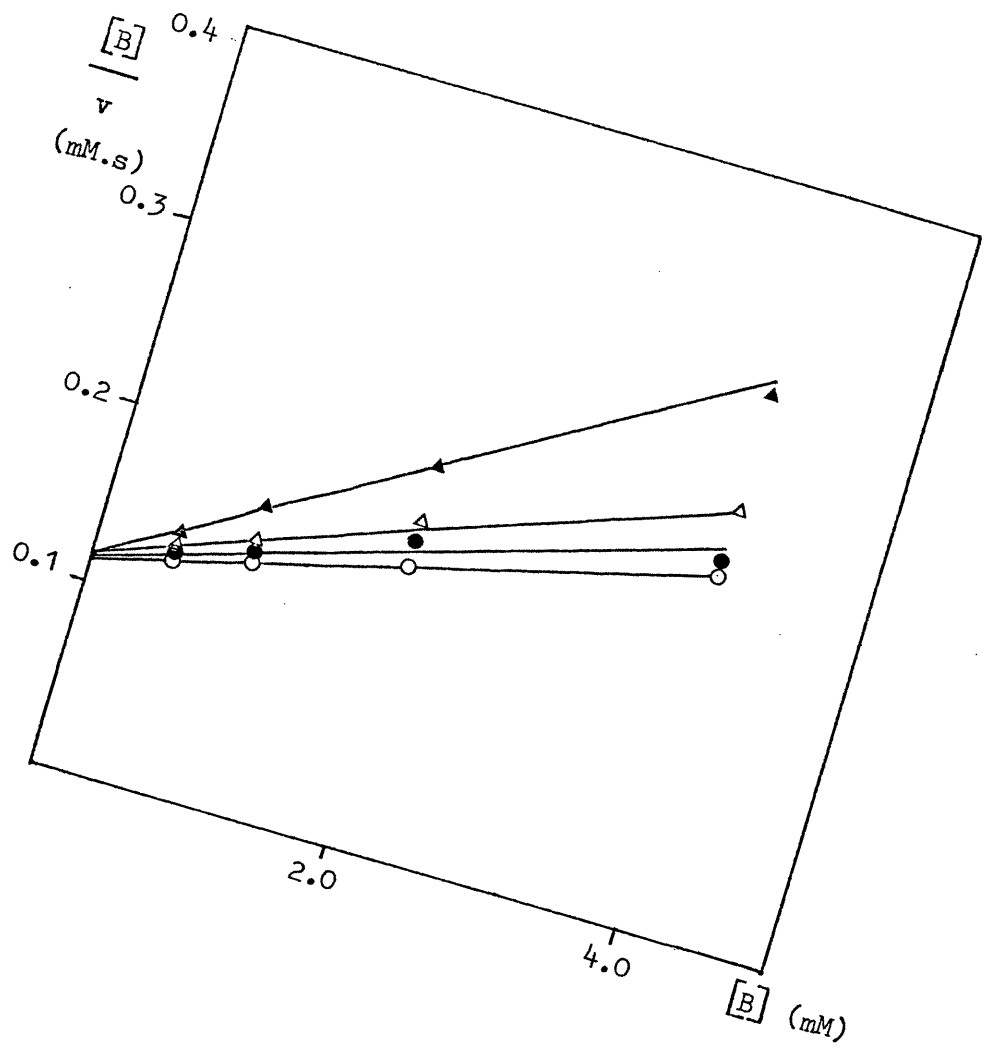
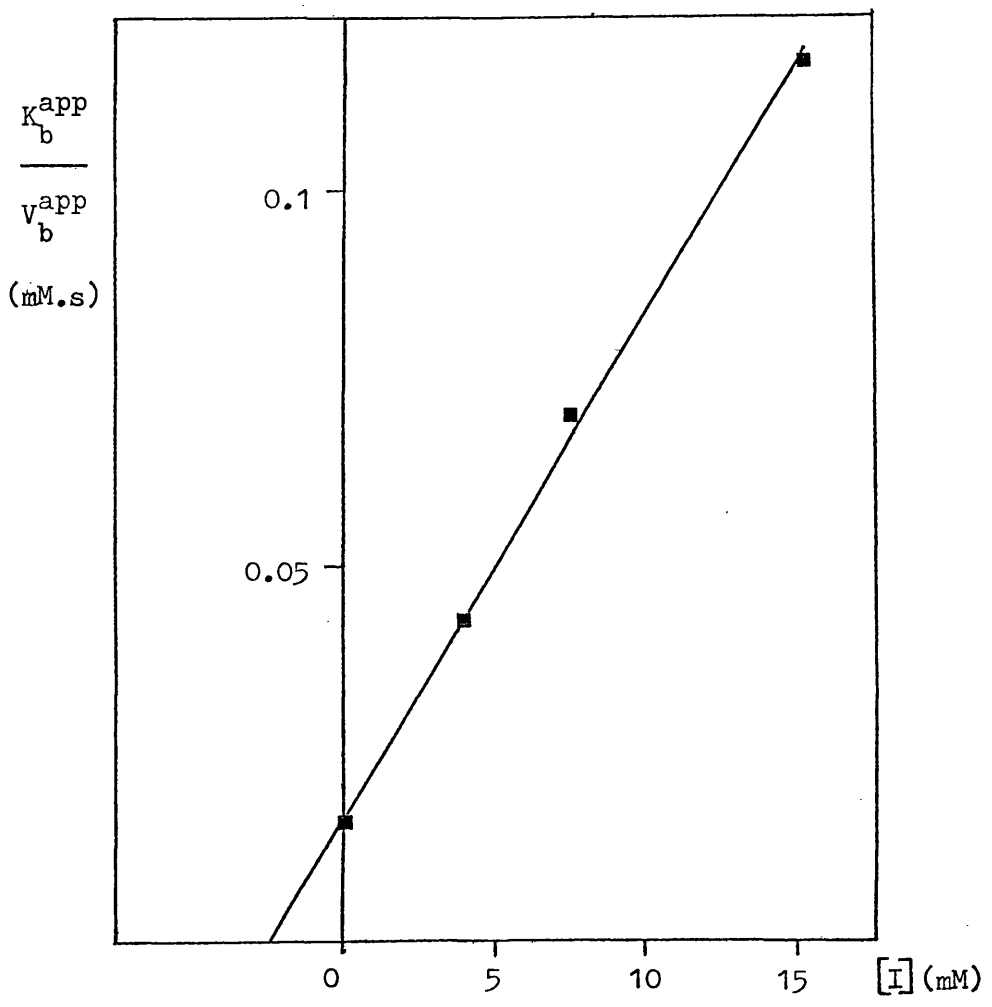

[B]
v
(mM.s)
0.4
0.3
0.2
0.1
2.0
4.0
[B] (mM)

Figure 27

Secondary plot of the effect of difluoro-aspartate concentration [I] on K_b^{app} / V_b^{app}.

Data points (■) are the mean of estimates of K_b^{app} / V_b^{app} obtained from studies on the variation of initial rate with respect to aspartate [B] concentration at the various 2-oxoglutarate [A] concentrations and at constant difluoro-aspartate. The line was drawn by linear regression analysis.


$\frac{K_b^{app}}{V_b^{app}}$ (mM.s)
0.1
0.05
0
5
10
15
[I] (mM)

doubt may be cast on the validity of this result since the estimated K_i' was some 3 fold higher than the highest difluoro-aspartate concentration (15mM) utilized, however linear regression was significant at 1%.

(3) iii). <u>The interaction of difluoro-aspartate with the aldimine form of AAT.</u>

As with the interaction of difluoro-aspartate with aminic AAT quantification of the interaction of the inhibitor with aldimine enzyme may be obtained from the effect of difluoro-aspartate on K_b^{app} / V_b^{app}. A plot of K_b^{app} / V_b^{app} versus [I] was similarily linear and yielded a value for K_i of 2.25mM, (Fig. 27). The relevant equation for this plot is obtained from equations (8) and (9):-

$$\frac{K_b^{app}}{V_b^{app}} = \frac{K_b\ (1 + [I]/K_i)}{V} \qquad \ldots(13)$$

(3) iv). <u>Anomalies in the variation of $1/V_a^{app}$ and $1/V_b^{app}$ with co-substrate and difluoro-aspartate concentration.</u>

Examination of equation (10) and (11) shows that K_i and K_i' may be also obtained from the uncompetitive element of the inhibition with respect to 2-oxoglutarate (A) and aspartate (B) respectively. The effect of difluoro-aspartate concentration [I] on V_a^{app} and V_b^{app} can provide this information and check the consistency of the data to equation (1) and thus the scheme of

Figure 17.

Rearrangement of equation (5) and (6) gives the following expressions respectively:-

$$\frac{[B]}{V_a^{app}} = \frac{K_b(1 + [I]/K_i)}{V} + \frac{1}{V}[B] \quad \ldots(14)$$

$$\frac{[A]}{V_b^{app}} = \frac{K_a(1 + [I]/K_i')}{V} + \frac{1}{V}[A] \quad \ldots(15)$$

Equations (14) and (15) predict for both plots of $[B]/V_a^{app}$ versus [B] (Fig. 28) and $[A]/V_b^{app}$ versus [A] (Fig. 29) at the various difluoro-aspartate concentrations respectively a family of parallel lines. This was not found to be the case, a family of converging lines was obtained for both plots. This phenomenon is indicative of a further element of uncompetitive inhibition. The simplest explanation consistent with the data is that difluoro-aspartate is capable of forming a non productive ternary$_{\wedge}^{complex}$ of the type E'AI or EBI. The rate equation corresponding to this addition to the scheme of Figure 17 is:-

$$v = \frac{V[A][B]}{K_a(1 + [I]/K_i')[B] + K_b(+ [I]/K_i)[A] + [A][B](1 + [I]/K_i'')} \quad \ldots(16)$$

Where K_i'' is the apparent dissociation constant of E'AI or EBI. The composition of this constant is mechanism dependent. Outlined in Figure 30 are possible mechanisms

Figure 28

Secondary plots of the effect of aspartate concentration [B] on V_a^{app} at the various difluoro-aspartate concentrations.

Plots of $[B]/V_a^{app}$ versus [B]. Values of V_a^{app} were obtained from the primary kinetic data as described in the text from studies in the variation of initial rate with respect to 2-oxoglutarate [A] concentration. Difluoro-aspartate concentraions: (●) 0, (o) 3.75, (▲) 7.5, (△) 15mM. The lines were drawn from parameters estimated from the data by direct linear plots of V_a^{app} against [B].

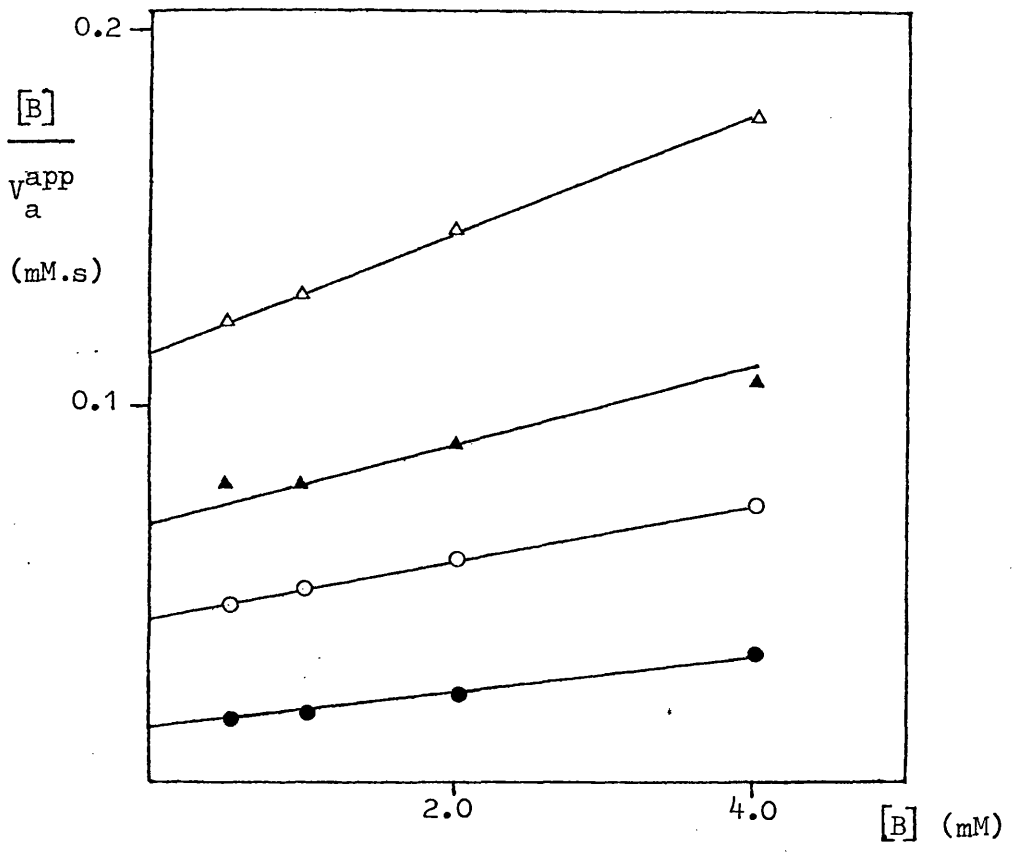

0.2
[B]
V_a^{app}
(mM.s)
0.1
2.0
4.0
[B] (mM)

Figure 29

Secondary plots of the effect of 2-oxoglutarate concentrations [A] on V_b^{app} at the various difluoro-aspartate concentrations.

Plots of $[A]/V_b^{app}$ versus [A] for the difluoro-aspartate concentrations (●) 0, (○) 3.75, (▲) 7.5, (△) 15mM. V_b^{app} was obtained from studies on the effect of aspartate [B] concentration on initial rate. The lines were drawn from parameters estimated from the data by the direct linear plot.

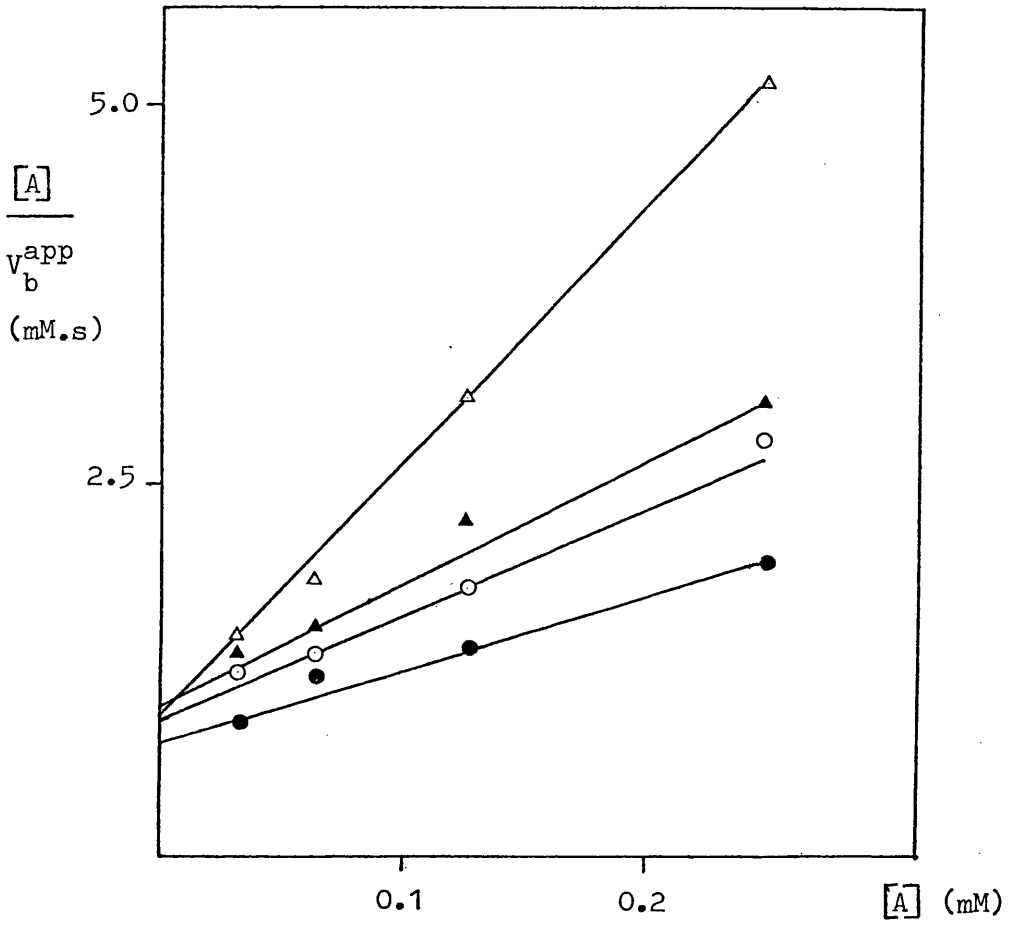

5.0
2.5
[A]
V_b^{app}
(mM.s)
0.1
0.2
[A] (mM)

Figure 30

Some simple mechanisms for the formation of the ternary enzyme-substrate-inhibitor complex.

(1) $E + B \rightleftharpoons EB \xrightarrow{k_4} E' + Q$

$E' + A \rightleftharpoons E'A \xrightarrow{k_2} E + P$

$E'A + I \overset{K}{\rightleftharpoons} E'AI$

$$K''_i = \frac{k_4}{k_4 + k_2} K$$

(2) $E + B \rightleftharpoons EB \xrightarrow{k_4} E' + Q$

$E' + A \rightleftharpoons E'A \xrightarrow{k_2} E + P$

$EB + I \overset{K}{\rightleftharpoons} EBI$

$$K''_i = \frac{k_2}{k_4 + k_2} K$$

(3) $E + B \rightleftharpoons EB \longrightarrow E' + Q$

$E' + A \rightleftharpoons E'A \longrightarrow E + P$

$E' + I \overset{K'_i}{\rightleftharpoons} E'I \quad . \quad E'I + A \overset{K}{\rightleftharpoons} E'IA$

$$K''_i = \frac{KK'_i}{K_a}$$

(4) $E + B \rightleftharpoons EB \longrightarrow E' + Q$

$E' + A \rightleftharpoons E'A \longrightarrow E + P$

$E + I \overset{K_i}{\rightleftharpoons} EI \quad . \quad EI + B \overset{K}{\rightleftharpoons} EIB$

$$K''_i = \frac{KK_i}{K_b}$$

Where E,E',A,B,P,Q,I,K_i,K'_i,K''_i,K_a,K_b, are as defined in Figure 17 and in text; and K the dissociation constant of the ternary enzyme-substrate-inhibitor complex as indicated.

for the formation of ternary enzyme-substrate-inhibitor complexes that would satisfy the data (and the composition of K_i'' relating to them). The form of the rate equation is independant of which of these complexes is formed (ie. E'AI or EBI). The rate equation is however different if significant amounts of both these complexes are formed. That this is probably not the case will be demonstrated. The parameters V_a^{app}, K_a^{app}, V_b^{app}, K_b^{app}, have now the new expressions:-

$$V_a^{app} = \frac{V\ [B]}{K_b(1 + [I]/K_i) + [B]\ (1 + [I]/K_i'')} \quad \ldots(17)$$

$$K_a^{app} = \frac{K_a(1 + [I]/K_i')\ \ [B]}{K_b(1 + [I]/K_i) + [B]\ (1 + [I]/K_i'')} \quad \ldots(18)$$

$$V_b^{app} = \frac{V\ [A]}{K_a(1 + [I]/K_i') + [A]\ (1 + [I]/K_i'')} \quad \ldots(19)$$

$$K_b^{app} = \frac{K_b(1 + [I]/K_i)\ \ [A]}{K_a(1 + [I]/K_i') + [A]\ (1 + [I]/K_i'')} \quad \ldots(20)$$

From these equations it can be shown that the ratios K_a^{app}/V_a^{app} and K_b^{app}/V_b^{app} are unaffected by this element of inhibition and so too the estimates of K_i' (Fig. 22) and K_i (Fig. 27) based on them. However, equations (17) and (19) now predict for plots of $[B]/V_a^{app}$ versus [B] and $[A]/V_b^{app}$ versus [A] at the various difluoro-aspartate concentrations the observed family of converging lines (Figs. 28 and 29 respectively).

Rearrangement of equations (17) and (19) for these plots give respectively:-

$$\frac{[B]}{V_a^{app}} = \frac{K_b(1 + [I]/K_i)}{V} + \frac{1 + [I]/K_i''}{V}\,[B] \quad \ldots(21)$$

$$\frac{[A]}{V_b^{app}} = \frac{K_a(1 + [I]/K_i')}{V} + \frac{1 + [I]/K_i''}{V}\,[A] \quad \ldots(22)$$

The ordinate intercepts (designated $K_{a,b}^{app} / V_{a,b}^{app}$) from the former plot (fig. 28) may be plotted against [I] (Fig. 31) to yield an estimate of K_i. A value of 2,75mM for K_i was thus obtained in close agreement with the K_i estimated as the competitive element of inhibition with respect to aspartate (Fig. 27); demonstrating the consistency of the data.

However it appears that the experimental error is greater than the small variation of ordinate intercept with [I] obtained in Figure 29, such that a converse plot cannot be used to obtain an estimate of K_i'. Examination of equation (21) and (22) shows that K_i'' may be estimated from a replot of the slopes of both Figures 28 and 29.

The plot of the slopes of Figure 28 versus difluoro-aspartate concentration was linear and gave a value for K_i'' of 5.9mM (Fig. 32). This linearity suggests that only

Figure 31

Tertiary plots of the effect of difluoro-aspartate concentration [I] on the ordinate intercepts of Figure 28.

Estimates of the intercepts (designated $\frac{K^{app}_{a,b,}}{V^{app}_{a,b,}}$) were obtained as described in Figure 28. The line was drawn by linear regression analysis.

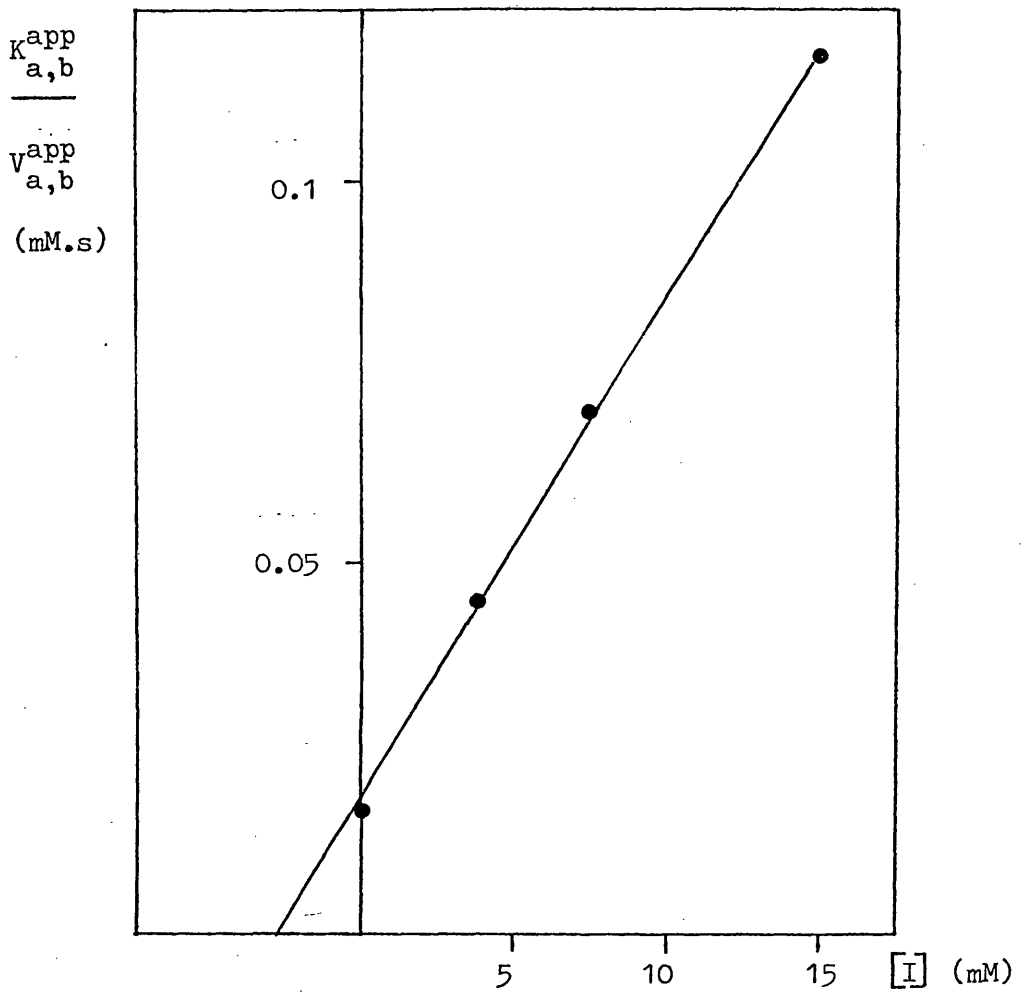
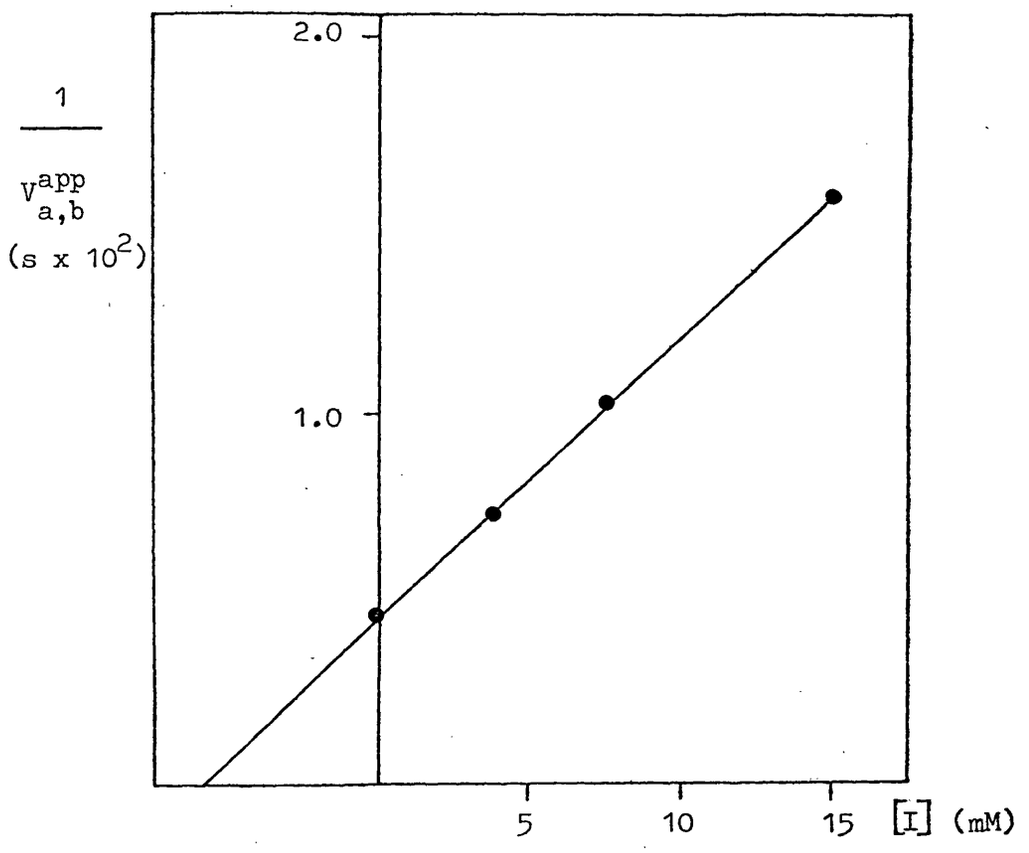

$K^{app}_{a,b}$
$V^{app}_{a,b}$
(mM.s)
0.1
0.05
5
10
15
[I] (mM)

Figure 32

Tertiary plot of the effect of difluoro-aspartate concentration [I] on the slope of Figure 28.

Estimates of the slopes of Figure 28 (designated $1/V^{app}_{a,b,}$) were obtained as described in that figure. The line was drawn by linear regression analysis.


2.0
1.0
1
$V^{app}_{a,b}$
(s x 10^2)
5
10
15
[I] (mM)

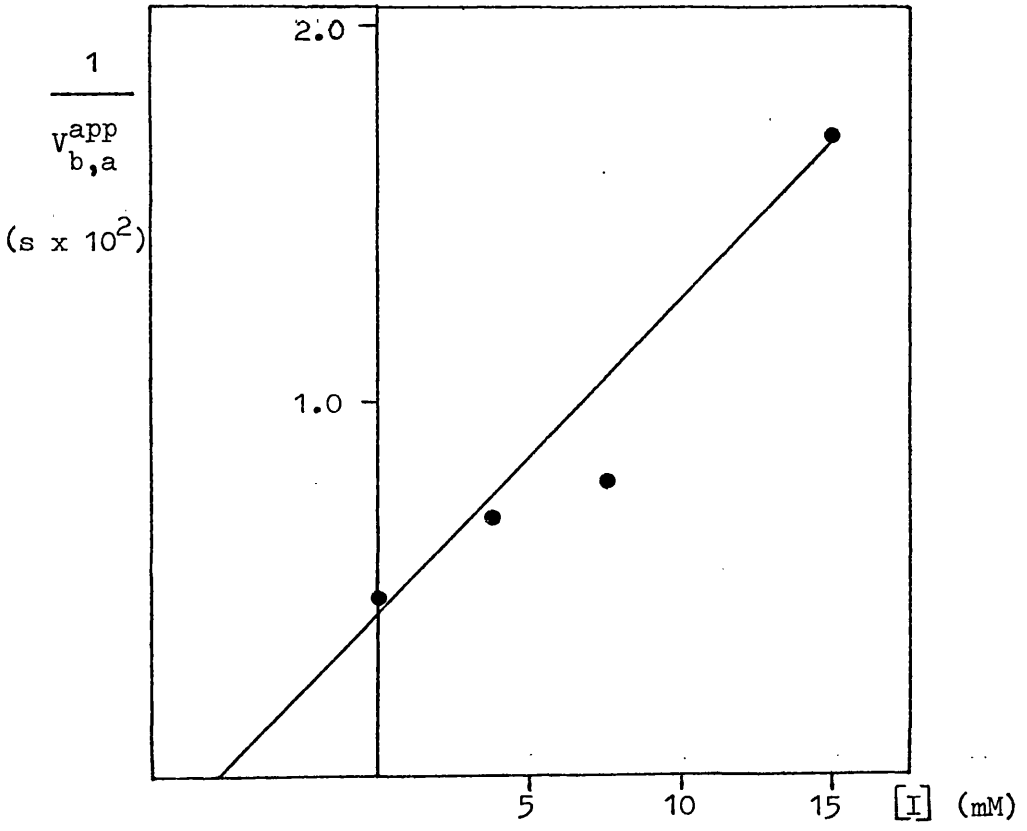
one of the ternary complexes E'AI and EBI is formed appreciably. If both these complexes were formed an $([I])^2$ term would be introduced into the relevant equation resulting in a parabolic variation of slope with difluoro-aspartate concentration.

The observed lack of linearity for the variation of the slopes of Figure 29 with difluoro-aspartate concentration (Fig. 33) could however accommodate a model in which both ternary complexes are formed. In view of the linearity of the previous plot (Fig. 32) and considering the relative quality of the primary kinetic data with respect to aspartate (Figs. 23-26) and 2-oxoglutarate (Figs. 18-21) it may rather reflect experimental error. With this in mind an estimate for K_i'' of 5mM was obtained by linear regression analysis.

Figure 33

Tertiary plot of the effect of difluoro-aspartate concentration [I] on the slopes of Figure 29.

Estimates of slope (designated $1/V_{b,a}^{app}$) were obtained from Figure 29 as described. The line was drawn by linear regression analysis, the datum point at [I] = 7.5mM was treated as an outlier.


1
$V^{app}_{b,a}$
(s x 10^2)
2.0
1.0
5
10
15
[I] (mM)

(4) Spectrophotometric Study of the Binding of Difluoro-Aspartate to the Aldimine form of Aspartate Aminotransferase.

Spectroscopic measurements of the coenzyme chromophore, either when free or when bound to the substrates or various pseudo-substrates, has provided useful information on the mechanism of this enzyme. This was therefore applied to the study of the interaction of difluoro-aspartate with the aldimine form of AAT.

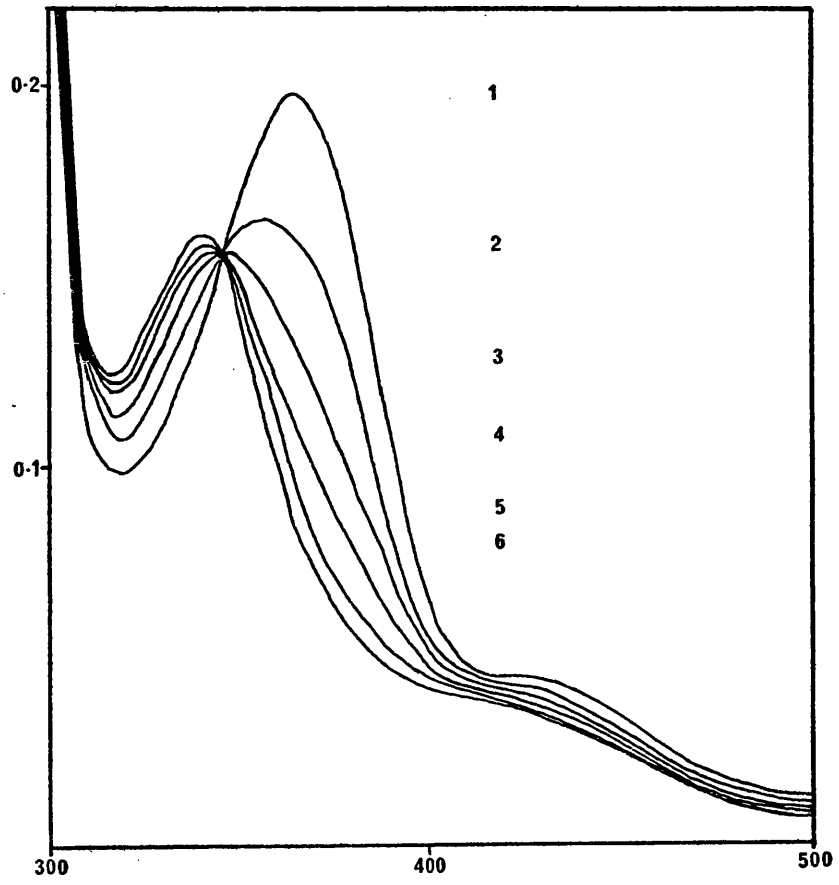
(4) i). Spectral changes resulting from the incremental addition of difluoro-aspartate to aspartate aminotransferase at pH 7.4.

The absorption spectrum of a solution (1ml) of the aldimine form of AAT (α-subform; 25.4μM, sites) in 100mM-pyrophosphate buffer, pH 7.4, contained in a 1cm pathlength semi-micro quartz cuvette at 25°C was recorded in the range 300-500nm. Aliquots (2 to a total 30 μl) of a 0.5M-difluoro-aspartate solution in 100mM-pyrophosphate buffer, pH 7.4, were added consecutively and the spectra recorded (Fig. 34). This incremental addition of difluoro-aspartate resulted in an instantaneous loss of the predominant absorbance band of AAT (363nm) and the formation of a new discrete maximum at 340 nm, indicative of complex formation.

Figure 34

Spectral changes on incremental addition of difluoro-aspartate to aspartate aminotransferase at pH 7.4.

1) Absorption spectrum of AAT (α-subform, aldimine form; 25.4 μM in 100mM-pyrophosphate buffer, pH 7.4) and in the presence of 2) 1.0mM; 3) 2.5mM; 4) 5.0mM; 5) 10.0mM; 6) 15.0mM-difluoro-aspartate. Spectra were corrected for the dilution on addition of difluoro-aspartate.


0·2
0·1
A
1
2
3
4
5
6
300
400
500
wavelength
(nm)

(4) ii). Determination of the dissociation constant and extinction coefficient of the aldimine-aspartate aminotransferase-difluoro-aspartate complex at pH 7.4.

The method of spectral titration permitted characterisation of this interaction under the following simple scheme:-

$$E + S \rightleftharpoons ES \qquad \ldots\ldots(1)$$

λmax 363 λmax 340

(E, aldimine-AAT; S, difluoro-aspartate; ES, enzyme-difluoro-aspartate complex with λ max 340nm).

The dissociation constant of the complex (ES) is written as:-

$$K = [E]\,[S] / [ES] \qquad \ldots\ldots(2)$$

If the total concentration of enzyme is [Et] then:-

$$[Et] = [E] + [ES]$$

Since [S] is always much greater than [Et] the concentration of difluoro-aspartate is taken as the total concentration.

If the absorbance of the enzyme in the absence of difluoro-aspartate is A_o and in the presence of difluoro-aspartate is A_s then :-

$$A_S = \varepsilon_E [E] + \varepsilon_{ES} [ES]$$

$$A_O = \varepsilon_E [Et]$$

where ε_E and ε_{ES} are the extinction coefficients of free enzyme and the enzyme-difluoro-aspartate complex (ES) respectively.

The change in absorbance (Δ A) on addition of

difluoro-aspartate is therefore equal to:-

$$\Delta A = \Delta A_O - \Delta A_S$$

$$\Delta A = \varepsilon_E [Et] - (\varepsilon_E [E] + \varepsilon_E [ES]) \quad .....(4)$$

Consideration of equations (2) and (3) yield the following expressions for [E] and [ES] :-

$$[E] = \left(\frac{[Et]}{1 + \frac{K}{[S]}} \right) \frac{K}{[S]} \quad .....(5)$$

$$[ES] = \frac{[Et]}{1 + \frac{K}{[S]}} \quad .....(6)$$

Substitution of equations (5) and (6) in equation (4) yields:-

$$\Delta A = \varepsilon_E [Et] - \left(\left(\frac{\varepsilon_E [Et]}{1 + \frac{K}{[S]}} \right) \frac{K}{[S]} + \frac{\varepsilon_{ES} [Et]}{1 + \frac{K}{[S]}} \right)$$

rearrangement gives:-

$$\Delta A = \frac{[Et] (\varepsilon_E - \varepsilon_{ES}) [S]}{K + [S]}$$

or

$$\frac{1}{\Delta A} = \frac{K}{[Et] (\varepsilon_E - \varepsilon_{ES})} \frac{1}{[S]} + \frac{1}{[Et] (\varepsilon_E - \varepsilon_{ES})} \quad .....(7)$$

Equation (7) predicts that for all wavelengths where ε_E differs significantly from ε_{ES} that plots of $1/\Delta A$ versus $1/[S]$ will be linear with a common abscissa intercept ($1/K$). Illustrated in Figure 35 are two plots taken from

absorption changes at 363nm and 340nm (the absorption maximum of E and ES respectively); these are the wavelengths at which K is most precisely determined. The linearity of these plots together with their common abscissa intercept (K = 2.5mM [1.5- 3.8] at 340nm and K = 2.5mM [2.1- 2.8] at 363nm) indicates adherence to the scheme of equation (1). The figures within square brackets are the calculated non-parametric 95% confidence interval for the parameter, this notation will be used throughout. For the plots of Figure 35 the line was drawn from the parameters $1/[Et](\varepsilon_E - \varepsilon_{ES})$ and $K/[Et](\varepsilon_E - \varepsilon_{ES})$ determined from the data by direct linear plots (Cornish-Bowden and Eisenthal, 1978) of $1/\Delta A$ against $[S]/\Delta A$.

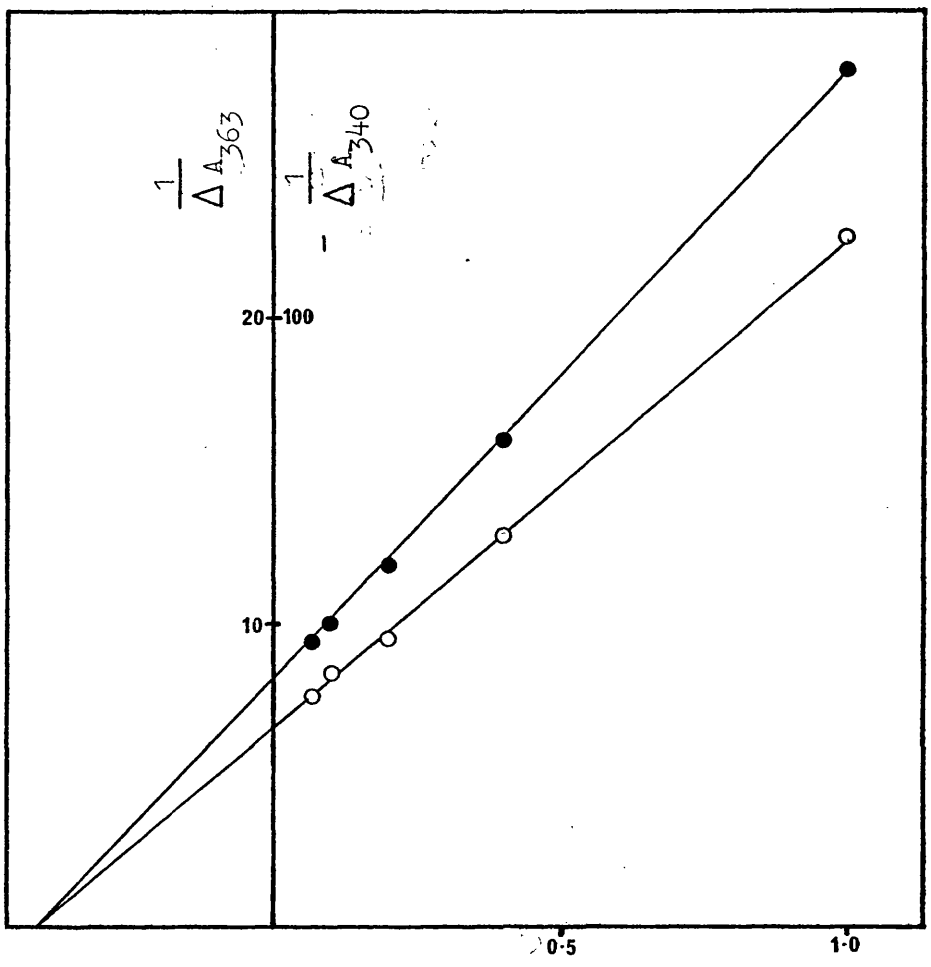
Similar direct linear plots of the data at 5nm intervals throughout the spectra permitted calculation of the spectrum of the aldimine-AAT-difluoro-aspartate complex, (Fig. 36). This arises from estimation of the parameter $1/[Et](\varepsilon_E - \varepsilon_{ES})$ at the various wavelengths with knowledge of the values [Et] and ε_E (Methods).

The extinction coefficient of the ES complex at its absorption maximum (340nm) was found to be 6.4×10^3 $M^{-1}.cm^{-1}$ [6.3 - 6.5] . To be noted also is the minor absorption band at ca 430nm of the ES complex.

Figure 35

Spectral titration curve for the binding of difluoro-aspartate to aspartate aminotransferase at pH 7.4.

Plots of $1/\Delta A$ (at 363nm (●) and at 340nm (○)) against $1/[S]$ from the data of Figure 34.

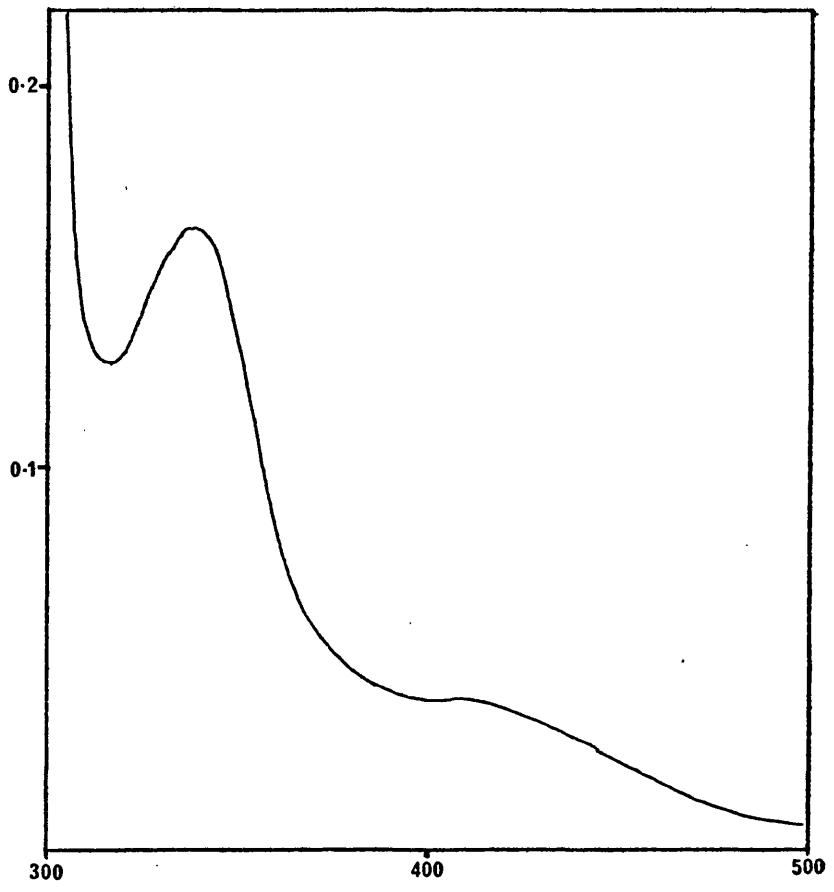


$\frac{1}{[S]}$

(mM^{-1})

Figure 36

Computed spectrum for the aldimine-aspartate aminotransferase difluoro-aspartate complex at pH 7.4.


A
0·2
0·1
300
400
500
wavelength
(nm)

(4) iii). Spectral changes resulting from the incremental addition of difluoro-aspartate to aspartate aminotransferase at pH 5.5.

AAT is known to undergo a pH dependent spectral change. At pH values below ca 6.3 enzyme with a protonated coenzyme absorbing at 430nm predominates. It was also therefore of interest to study the interaction of difluoro-aspartate with AAT at pH 5.5.

In a manner identical to the titration at pH 7.4, aliquots (5 to a total of 40 µl) of 0.5M-difluoro-aspartate (in 100mM-pyrophosphate buffer, pH 5.5) were added to a solution of aldimine-AAT (α-subform; 28.9 µM in 100mM-pyrophosphate buffer, pH 5.5) and the spectra recorded (Fig. 37).

Addition of difluoro-aspartate resulted in an instantaneous loss in absorbance at 430nm and an increase in absorbance at 340nm.

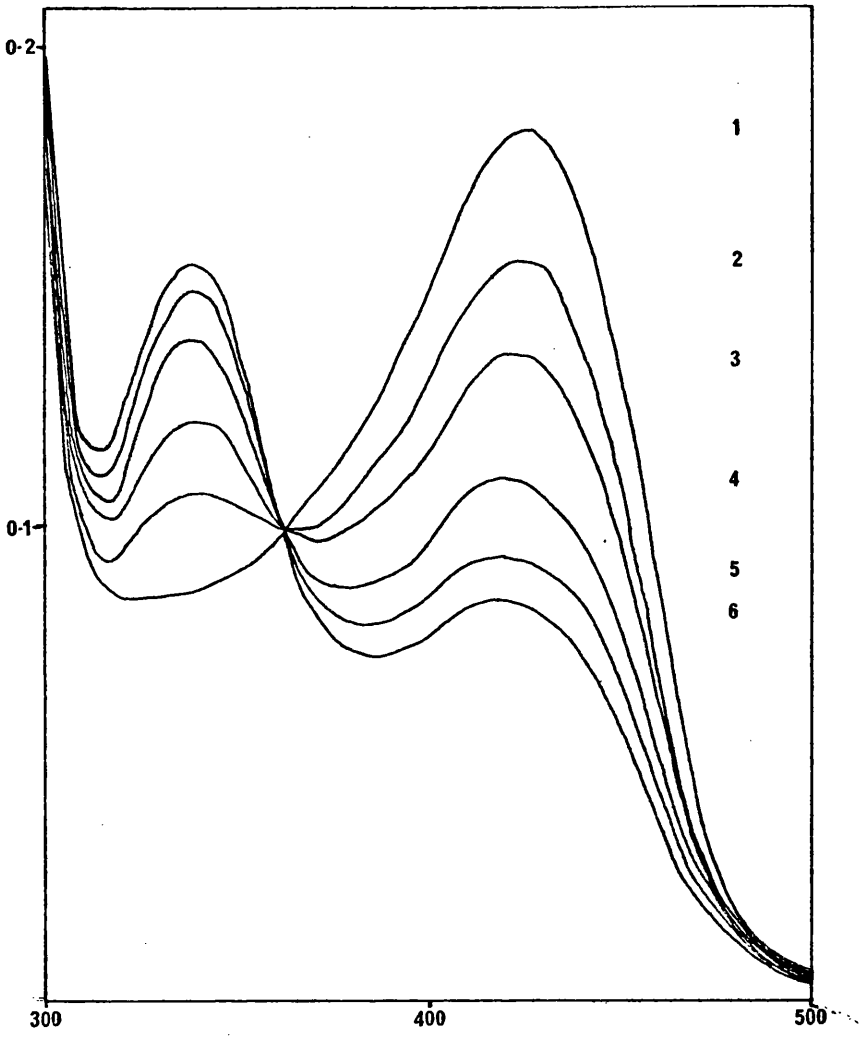
(4) iv). Determination of the dissociation constant and extinction coefficient of the aldimine-aspartate-aminotransferase-difluoro-aspartate complex at pH 5.5.

An identical theory and method as applied to the titration at pH 7.4 in section 4.ii may also be applied at pH 5.5. Thus values for K at 430nm of 12.1mM [10.2 - 16.2] and at 340nm of 10.6mM [9.7 - 12.1] were found

Figure 37

Spectral changes on incremental addition of difluoro-aspartate to aspartate aminotransferase at pH 5.5.

1) Absorption spectrum of AAT (α-subform, aldimine form; 28.9 μM in 100mM-pyrophosphate buffer, pH 5.5) and in the presence of 2) 2.5mM; 3) 5.0mM 4) 10.0mM; 5) 15.0mM; 6) 20.0mM-difluoro-aspartate.

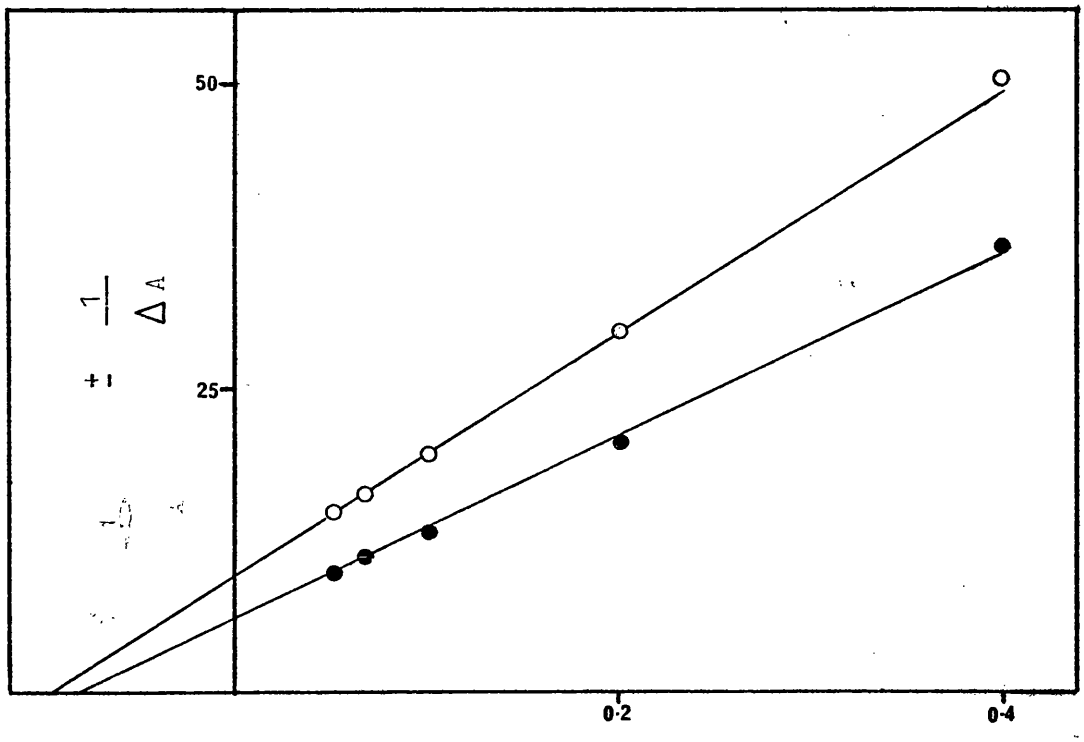

A
0·2
0·1
300
400
500
1
2
3
4
5
6
wavelength
(nm)

(Fig. 38). The spectrum of the complex was also computed (Fig. 39) and εES_{340} found to be 6.5×10^3 $M^{-1}.cm^{-1}$ [6.4 - 6.8] . The spectrum of the ES complex again exhibited an absorption maximum at 340nm with a minor band at 430 nm.

Figure 38

Spectral titration curve for the binding of difluoro-aspartate to aspartate aminotransferase at pH 5.5.

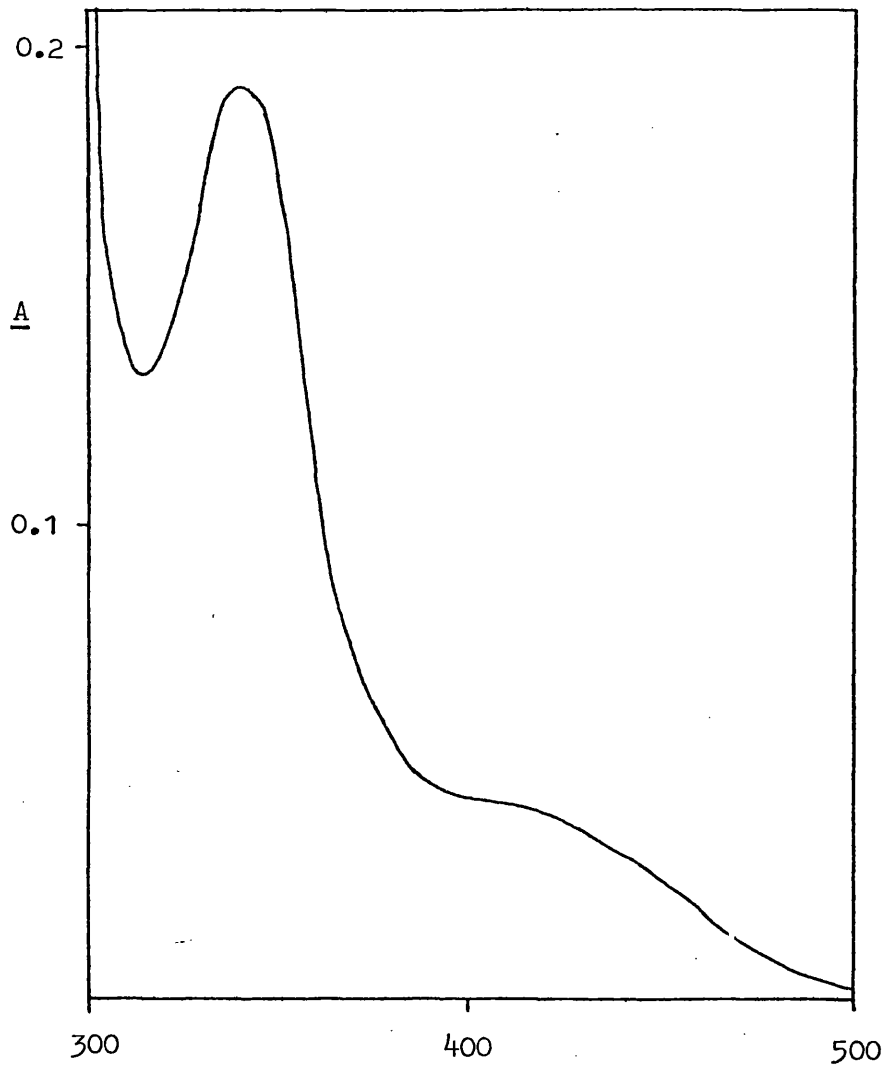
Plots of $1/\Delta A$ (at 430nm (●) and at 340nm (○)) against $1/[S]$ from the data of Figure 37.



$\frac{1}{[S]}$ (mM^{-1})

Figure 39

Computed spectrum for the aldimine-aspartate aminotransferase-difluoro-aspartate complex at pH 5.5.


0.2
A
0.1
300
400
500
wavelength
(nm)

(4) v.) Variation of the dissociation constant of the aldimine-aspartate-aminotransferase-difluoro-aspartate complex with pH.

The results of the foregoing spectral study on the interaction of difluoro-aspartate with AAT indicated a pH variation of the dissociation constant (K) of the complex. The pH variation of this parameter was therefore studied in more detail using the mixed subforms of AAT; this was justified on the basis that the measured values of K at pH 5.5 and pH 7.4 are not significantly different for the mixed and α-subforms viz. pH 7.4, α-subform K = 2.5 [2.1-2.8] , mixed-subforms K = 2.6 [1.6-3.2] ; pH 5.5, α-subform K = 12.1 [10.2-16.3] , mixed-subforms K = 10.7 [8.7-12.9] .

The extinction coefficient at 340nm ($\varepsilon_{ES,340nm}$) and the dissociation constant (K) of the enzyme-difluoro-aspartate complex were determined as described previously (4.ii) by spectral titrations in 100mM-pyrophosphate buffer.

$\varepsilon_{ES,340nm}$ was again found to be invariant with pH (Table 4); however the values found were significantly different from the values found for the α-subform complex (Table 4).

The variations of K determined from changes in absorbance at 360nm and 430nm and at 340nm with pH are presented in Figures 41i and 41ii respectively. The 'bell shaped' profile may be analysed under the following scheme

from the precedent set by Fasella et al., (1966) in their study of the pH variation of the binding of α-methylaspartate to AAT with the inclusion of amino-acid ionization:-

$$\begin{array}{ccccc} H^+ + E & + & SH & \overset{K^o}{\rightleftharpoons} & ESH \\ K_{a,a} \updownarrow & & \updownarrow & & K_{a,s} \\ EH & & S + H^+ & & \end{array}$$

(where $K_{a,a}$ and $K_{a,s}$ are the acid dissociation constants of AAT are difluoro-aspartate respectively; K^o is the pH-independent dissociation constant of the enzyme-difluoro-aspartate complex).

Adjustment of the measured dissociation constant (K) for the concentration of reactive zwitterionic difluoro-aspartate (pK = 7.18; Fig. 14) gives the curve (Fig. 41i). Hence the zwitterionic-difluoro-aspartate-AAT dissociation constant (K') is related to K^o by the expression:-

$$K' = K^o(1 + [H^+] / K_{a,a})$$

Thus a plot of K' versus $[H^+]$ is linear (Fig. 41ii) and allows determination of K^o = 0.9mM and $K_{a,a} = 3.3 \times 10^{7}$M ($pK_{a,a}$ = 6.5)

Table 4

Variation of the extinction coefficient ($\varepsilon_{ES,340}$) at 340nm of the AAT-difluoro-aspartate complex with pH.

pH	$\varepsilon_{ES,340}$. $M^{-1}.cm^{-1}$.
Mixed-subform-AAT	
5.50	7.4×10^3 [6.9-7.6]
5.75	7.1×10^3 [6.8-7.2]
6.10	7.1×10^3 [6.9-7.1]
6.80	7.3×10^3 [6.9-7.5]
7.00	7.0×10^3 [6.9-7.4]
7.10	7.2×10^3 [6.9-7.8]
7.40	7.5×10^3 [7.4-8.1]
8.00	7.0×10^3 [6.7-7.3]
* Weighted mean	7.2×10^3
α -subform-AAT	
5.50	6.5×10^3 [6.4-6.9]
7.40	6.4×10^3 [6.3-6.5]
* Weighted mean	6.4×10^3

* The weighting factor used was the reciprocal of the 95% confidence interval.

Figure 40i

Variation of the dissociation constant (K) of the aldimine-AAT-difluoro-aspartate complex with pH.

K was determined as described in text from changes in absorbance at 360nm (●) and at 430nm (○).

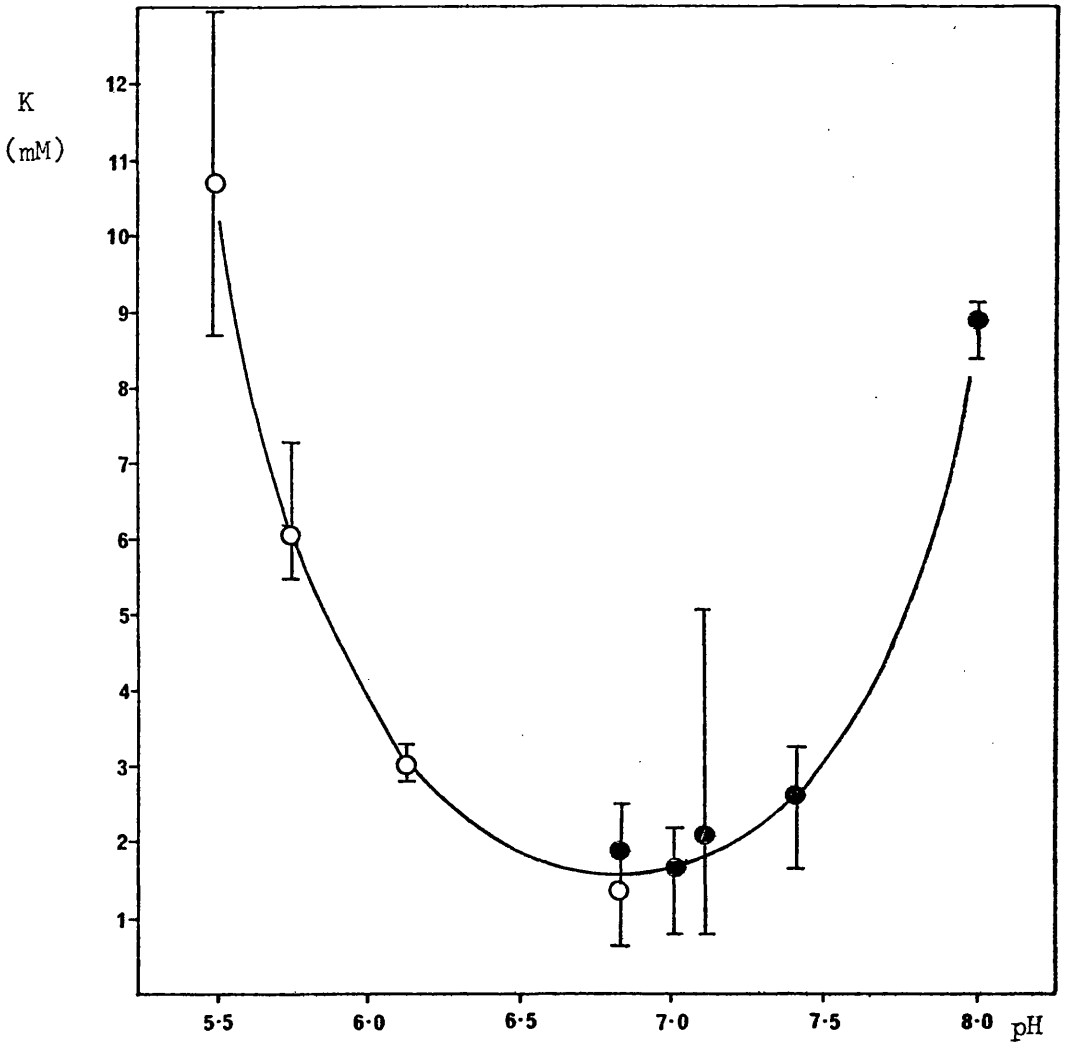

K
(mM)
12
11
10
9
8
7
6
5
4
3
2
1
5·5
6·0
6·5
7·0
7·5
8·0
pH

Figure 40ii

Variation in the dissociation constant (K) of the aldimine-AAT-difluoro-aspartate complex with pH.

K was determined as described in text from changes in absorbance at 340nm.

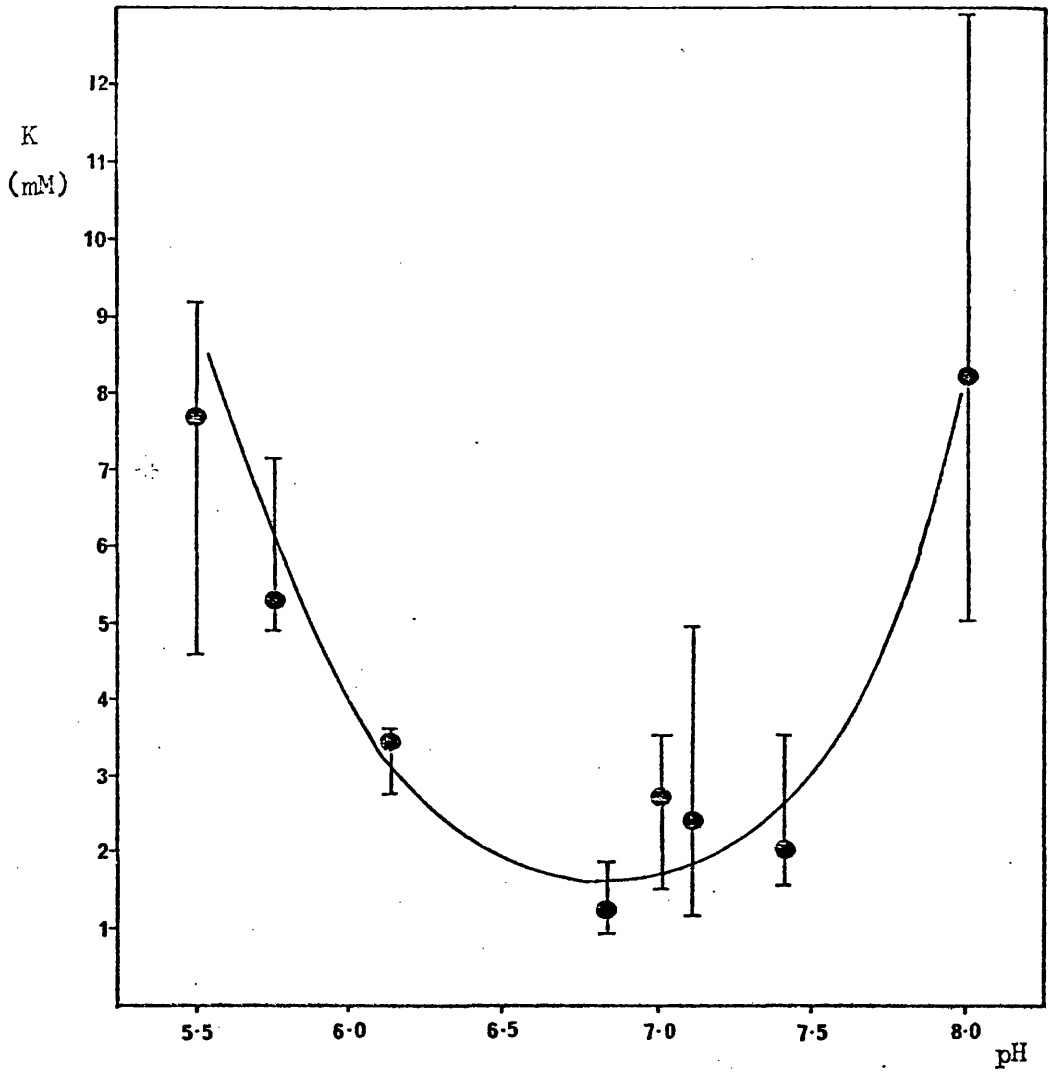

K
(mM)
12
11
10
9
8
7
6
5
4
3
2
1
5·5
6·0
6·5
7·0
7·5
8·0
pH

Figure 41i

Variation in the dissociation constant (K') of the aldimine-AAT-difluoro-aspartate complex with pH.

K' was obtained from the data of Figure 40i by correction of the measured dissociation constant (K) for the assumption that only the zwitterionic form of difluoro-aspartate ($pK_{a,s}$ = 7.18) was capable of interacting with AAT.

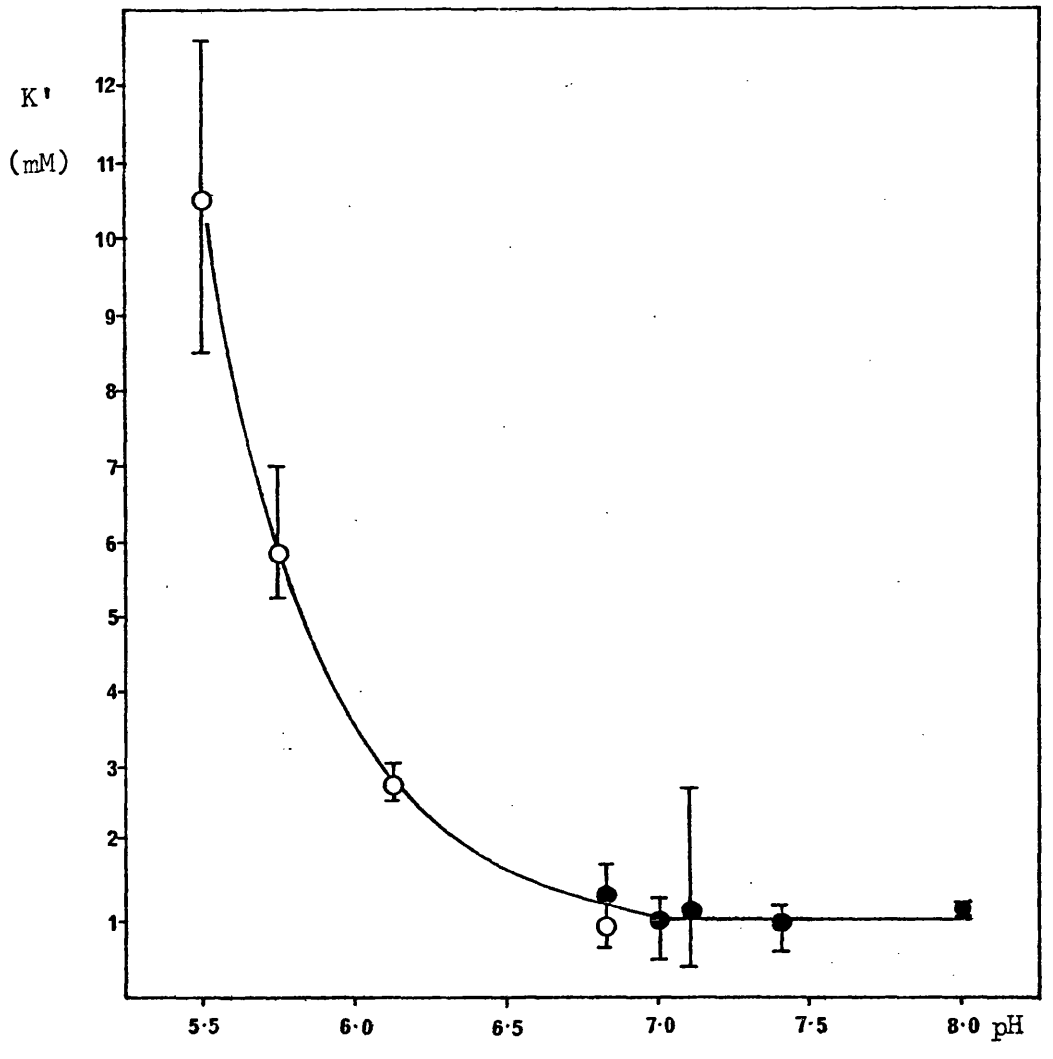
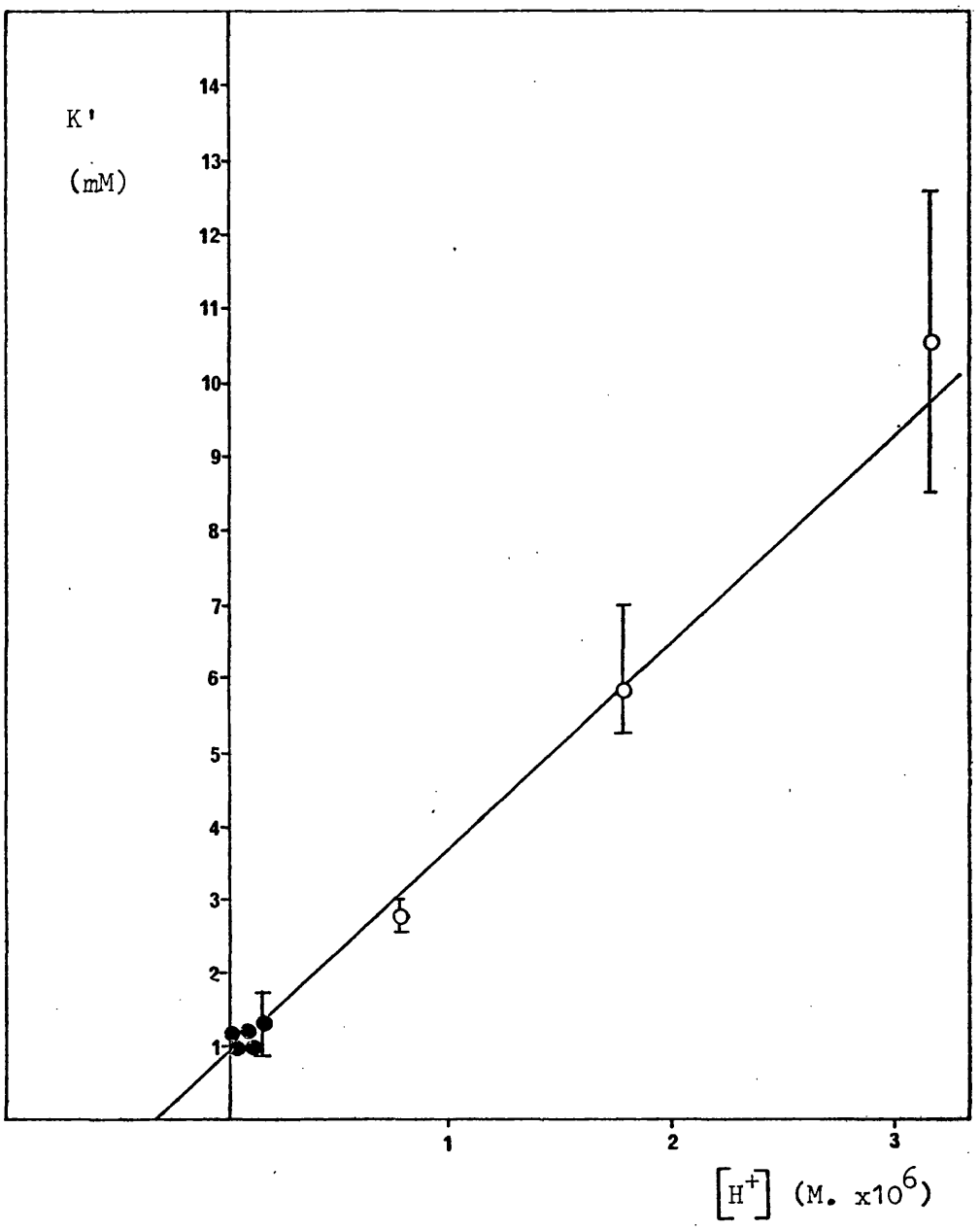

K'
(mM)
12
11
10
9
8
7
6
5
4
3
2
1
5·5
6·0
6·5
7·0
7·5
8·0
pH

Figure 41ii

Variation in the dissociation constant (K') of the aldimine-AAT-difluoro-aspartate complex with hydrogen ion concentration.

Plot of K' (taken from Fig. 41i) versus hydrogen ion concentration $\left[H^{+}\right]$.

The line was drawn by linear regression, the datum points were weighted according to the reciprocal of the 95% confidience interval.


K'
(mM)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
[H+] (M. x10^6)

(4) vi). Stopped-flow study of the binding of difluoro-aspartate to the aldimine form of aspartate aminotransferase.

Within the time scale of direct spectrophotometry the reaction of difluoro-aspartate with AAT appeared instantaneous. Therefore an attempt was made to study the kinetics of the formation of the difluoro-aspartate-AAT complexes by stopped-flow spectrophotometry.

The method of stopped-flow spectrophotometry in essence relies upon the rapid mixing of the reactants so that spectrophotometric observations may be made on the reaction with a time scale in the order of ms.

The α-subform of aldimine-AAT (128 μM in 100mM-pyrophosphate buffer, pH 7.4) contained in one syringe of a Durrum-Gibson stopped-flow spectrophotometer was rapidly mixed with an equal volumne (0.3ml) of a difluoro-aspartate solution (ranging in concentration from 2.5mM to 40mM in 100mM-pyrophosphate buffer, pH 7.4) contained in the other syringe. On cessation of flow (by a stopping-syringe) changes in absorbance at 370 nm were observed in the 2cm observation tube and displayed on a Tectronix storage oscilloscope.

The choice of 370nm was based on the difference spectrum between aldimine-AAT and the enzyme-difluoro-aspartate complex which shows a maximum at this wavelength (Fig. 42). This difference spectrum was obtained in the

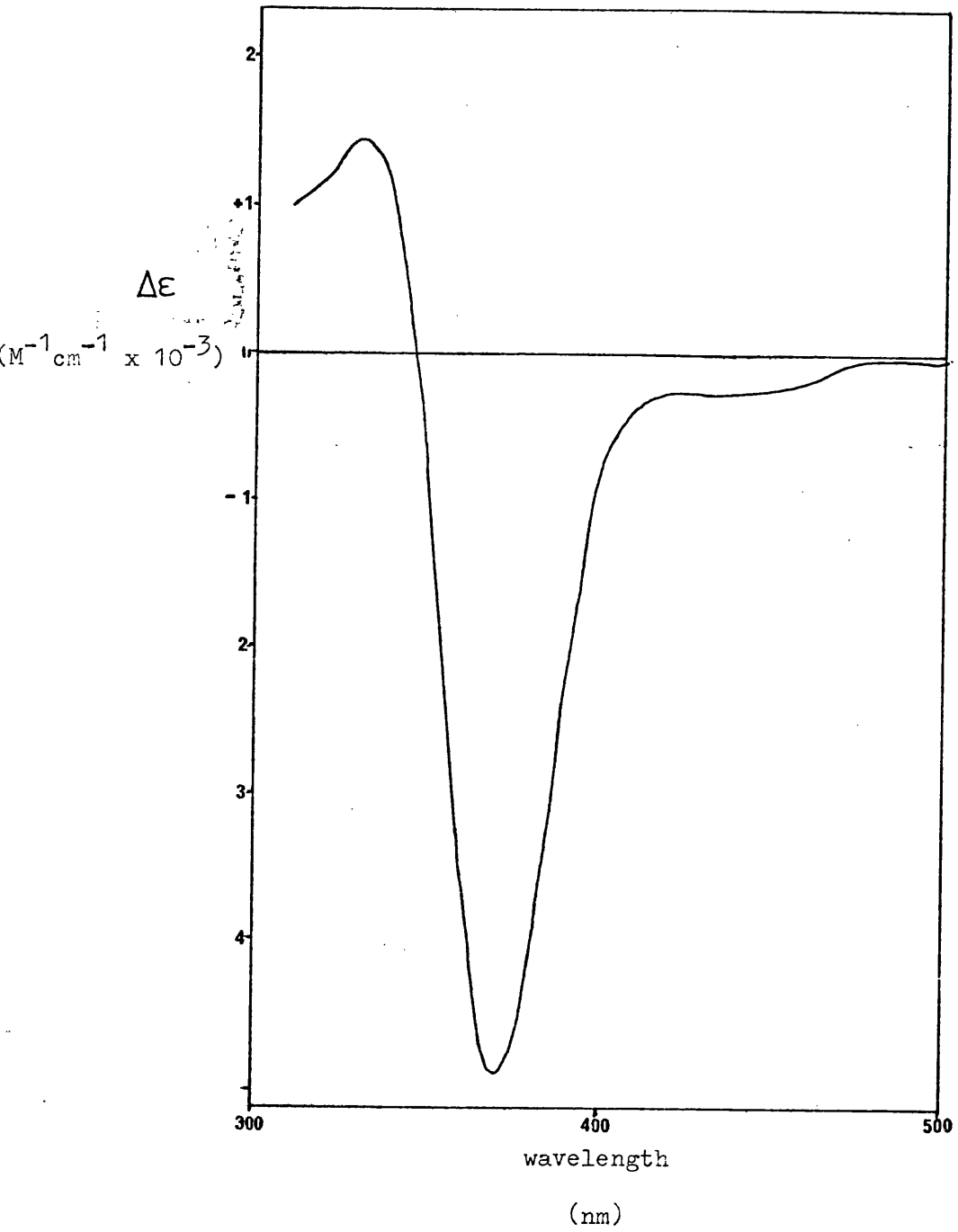
following manner. α-Aldimine-AAT (73 μM in 100mM-pyrophosphate buffer, pH 7.4) was titrated stepwise with difluoro-aspartate and the consecutive spectra recorded whilst the spectrophotometric blank comprised an identical enzyme solution alone. The difference in extinction coefficient ($\Delta\varepsilon$) between the enzyme-difluoro-aspartate complex and free enzyme was then computed from the data in a manner identical to that used to calculate the spectrum of the complex as described in Section 4 (ii), from direct linear plots of $[S]/\Delta A$ against $1/\Delta A$ where ΔA is the observed difference in absorbance between the enzyme plus difluoro-aspartate and enzyme alone. By this method the difference in extinction at 370nm was found to be 4.88 $M^{-1}.cm^{-1}$. The dissociation constant of the enzyme-difluoro-aspartate complex determined from the data at 370nm by this method was found to be 2.6mM.

Within the concentration range of difluoro-aspartate studied (ie. final concentration 1.25mM to 20mM) it was found that the reaction was so rapid as to preclude observations of the formation of the difluoro-aspartate-AAT complex. The reaction proceeded to equilibrium within the dead time of the instrument (3ms, C. Wharton personal communication) such that the optical density trace was continuous with the previous 'spent' shot.

However this result is not totally barren of information as it maybe used to provide some measure of

Figure 42

Difference spectrum for the aspartate aminotransferase-difluoro-aspartate complex.


Δε
(M⁻¹cm⁻¹ x 10⁻³)
2
+1
1
-1
2
3
4
300
400
500
wavelength
(nm)

the lower bounds for the apparent second order rate constant and the first order rate constant for the association and dissociation respectively of AAT and difluoro-aspartate. From the difference spectrum it can be shown that the mixing of the AAT solution with an equal volume of difluoro-aspartate solution (giving a final concentration of 64 μM-AAT and 1.25 mM-difluoro-aspartate) in the 2cm observation tube would result in an absorbance change of 0.284 at 370nm. It would not be unreasonable to assume that for an oscilloscope scale of 0.1 OD/cm an absorbance change of 0.017 (representing 6% of the total change) could be undiscernable. Thus the reaction can be considered to have reached 94% equilibrium within the dead time of the instrument. Now lack of any evidence to the contrary permits analysis of the reaction under the simple scheme for the spectrophotometric titration.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

According to the above scheme the approach to equilibrium starting from a free enzyme should follow a first order time course if the concentration of difluoro-aspartate is much greater than that of enzyme. A lower bound for the apparent pseudo first order rate constant apertaining to this process (k') may be obtained from the foregoing assumption on the undiscernability of the extent of completion of the reaction.

$$k' = \ln 2 / t_{\frac{1}{2}}$$

where the half life ($t_{\frac{1}{2}}$) of the reaction is such that 94% of the reaction is completed in the dead time of the

instrument (ie. 4 ($t_{\frac{1}{2}}$) ≙ 3 ms). The value of k' thus found is 924 s^{-1} and is related to the substrate concentration by the expression

$$k' = k_{-1} + k_1 [S] \qquad \ldots\ldots(8)$$

where k_{-1} is the first order rate constant for the dissociation of ES and k_1 the second order rate constant for the formation of ES. The previously determined spectral dissociation constant of ES (K) is given by the ratio

$$K = \frac{k_{-1}}{k_1} \qquad \ldots\ldots(9)$$

Therefore k_1 and k_{-1} may be found from the following substitutions of equation (9) into equation (8) with rearrangement

$$k_{-1} = \frac{k'}{(1 + [S]/K)}$$

$$k_1 = \frac{k'}{(K + [S])}$$

Thus with a value for K of 2.5mM (Fig.35) and with the lowest concentration of difluoro-aspartate (1.25 mM) a lower bound for the second order rate constant for the formation of ES (k_1) of 2.46 x $10^5 M^{-1}s^{-1}$ is found. The lower bound for the first order rate constant for the dissociation of ES (k_{-1}) is 616 s^{-1}.

(5) Borohydride Reduction of the Difluoro-aspartate Aspartate Aminotransferase complex with λmax 340nm.

The possibility exists that difluoro-aspartate reacts to form a Schiff base with the coenzyme of AAT. Such Schiff bases have been identified (Riva et al., 1964; Malakhova and Torchinskii, 1965) by reduction with borohydride to form a stable secondary amine, followed by resolution from the enzyme and electrophoretic comparison with the synthetic pyridoxal derivative. An attempt was made to follow this protocol in order to identify the AAT-difluoro-aspartate complex.

(5) i). Spectral changes on the addition of sodium borohydride to the aspartate-aminotransferase-difluoro-aspartate complex

The spectrum of a solution (1ml) of AAT (868 μM) in 100mM-pyrophosphate buffer, pH 7.4, was recorded in a 2mm path length cuvette (Fig. 43). Solid difluoro-aspartate (4mg) was added, bringing the concentration of difluoro-aspartate to 19.6mM. The resulting spectrum exhibited the characteristic absorption maximum at 340nm indicative of the formation of the enzyme-difluoro-aspartate complex. To this was added solid sodium borohydride (1.5mg $NaBH_4$ + 0.5mg NaB^3H_4, 332 mCi/mM) and after 20m the spectrum was recorded. The addition of sodium borohydride resulted in the loss of the 340nm absorbance and the formation of a new discrete maximum at

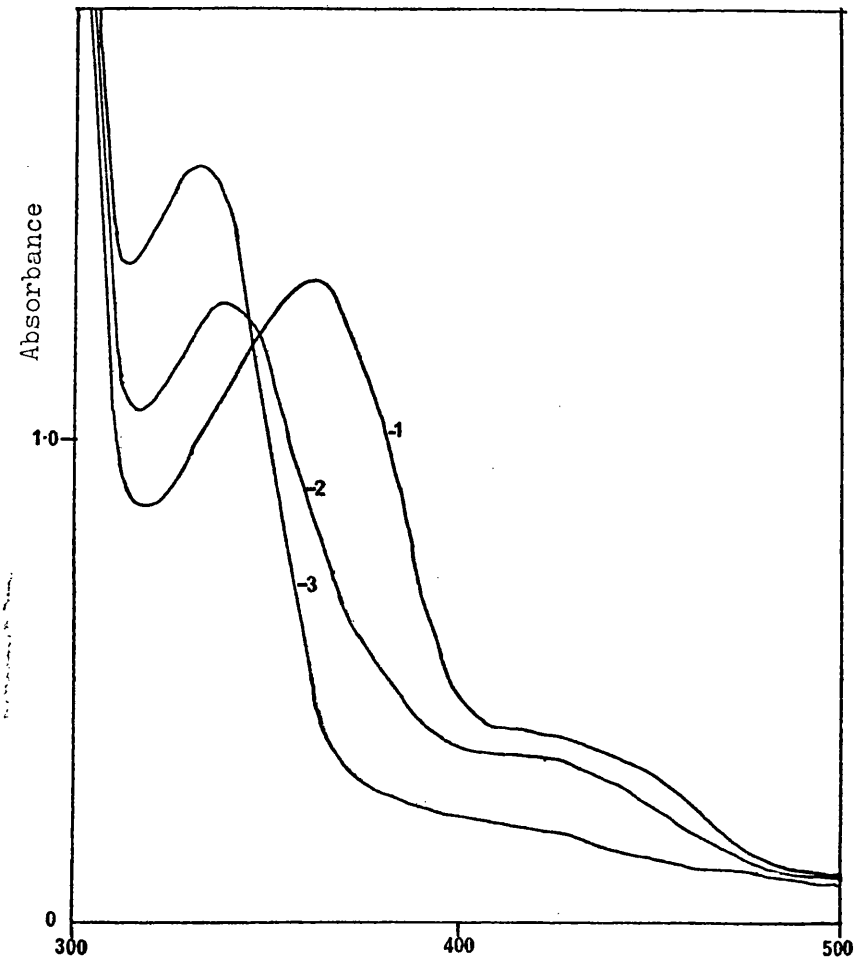
Figure 43

Spectral changes on the consecutive addition of difluoro-aspartate and sodium borohydride to aldimine aspartate aminotransferase.

1). Absorption spectrum of AAT, (868 μM; 100mM-pyrophosphate buffer, pH 7.4; 2mm path length cuvettes).

2). The same as above after the addition of solid difluoro-aspartate acid (4mg).

3). As 2, 20 m after the addition of solid sodium borohydride (1.5mg $NaBH_4$ + 0.5mg NaB^3H_4).


Absorbance
1·0
0
300
400
500
-1
-2
-3
wavelength
(nm)

ca 333nm (Fig. 43). This form of the enzyme was found to be enzymatically inactive.

5) ii). Attempted isolation of the reduced coenzyme-difluoro-aspartate complex.

Excess reagents were removed from the sodium borohydride reduced enzyme by passage through a column (30cm x 1.5cm) of Sephadex G25 equilibrated with 100mM-NaCl (Fig. 44). The column was eluted at 20ml/h with the same medium and fractions (2.6ml) were collected (Fig. 44). The absorbance of the eluant at 280 nm was monitored in a flow cell. Radioactivity in the fractions was determined by counting an aliquot (20 µl) in a scintillation cocktail (5ml) comprising 70% toluene/30% Triton X-100/0.5% PPO with a Packard Tri-Carb scintillation counter. No correction for quenching was made because all radioactivity determinations were made with the same volume of aqueous sample (20 µl). The total activity applied to the column was 2.3×10^8cpm. the combined excluded protein fractions (7-10) exhibited an identical spectrum to that of product (3) of Figure 43 and had a count of 9.23×10^6cpm.

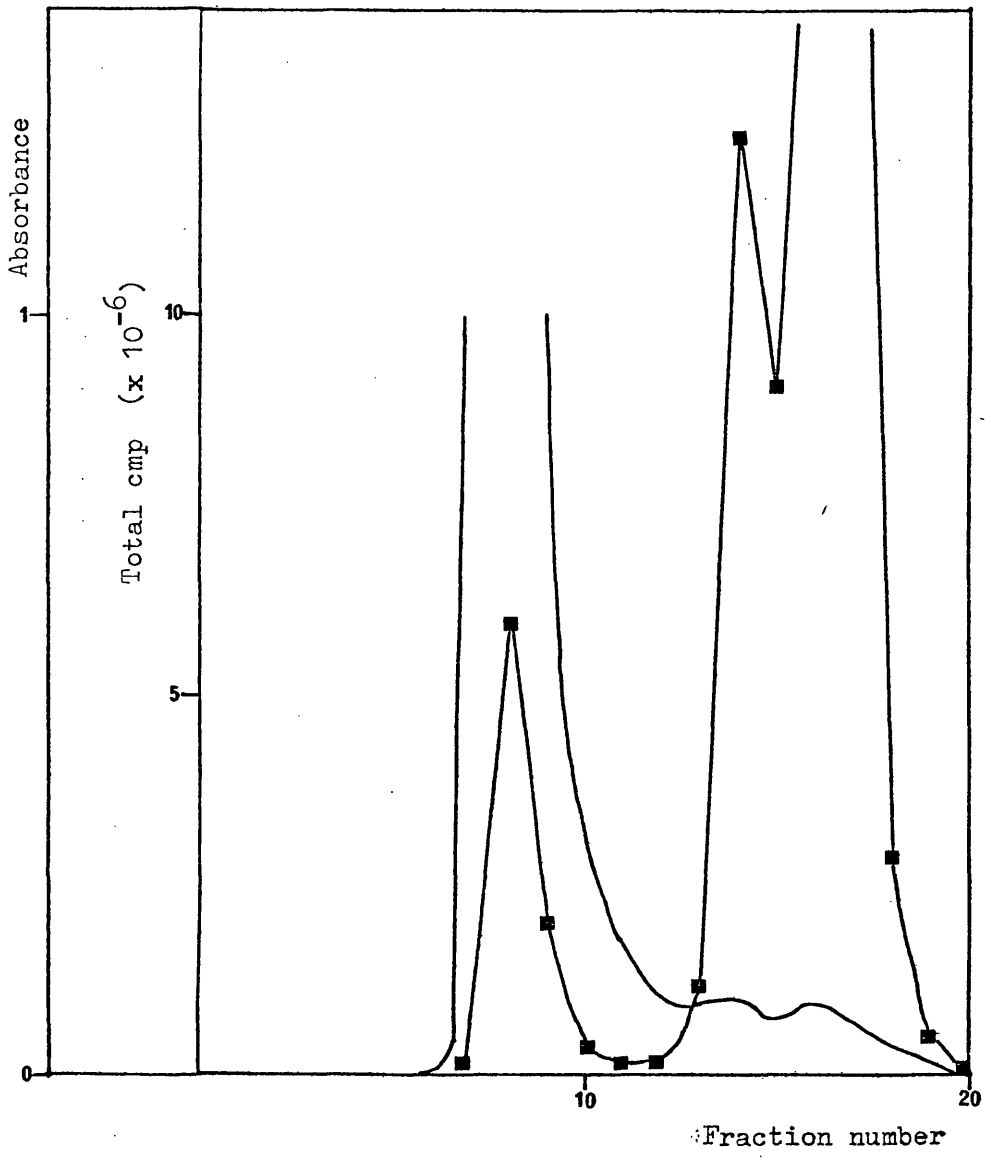
In order to resolve the reduced difluoro-aspartate-coenzyme complex from the enzyme the method employed was essentially that of Malakhova and Torchinskii (1965).

The excluded protein fraction was brought to pH 11

Figure 44

Gel filtration of the difluoro-aspartate and sodium borohydride treated aspartate aminotransferase.

The sodium borohydride reduction product of Figure 43, (1ml) was applied to a column (1.5cm x 30cm) of Sephadex G25 equilibrated and eluted with 100mM-NaCl. Absorbance at 280nm (——) and radioactivity (—■—) in the eluant was determined as described in the text.


Absorbance
1
0
Total cmp (x 10^{-6})
10
5
10
20
Fraction number

by addition of concentrated ammonia solution and left for 20m at room temperature. Protein was precipitated by the addition of acetone. After centrifugation 1.43×10^5 cpm, representing 1.55% of the total protein radioactivity was in the acetone supernatant. This represents the portion of coenzyme resolved from the enzyme.

In order to further attempt resolution, the acetone protein precipitate was resuspended in 100mM-pyrophosphate buffer, pH 7.4, (1ml) and clarified by centrifugation. This resulted in a solution containing 1.35×10^6cpm. representing 14.68% resuspension of total protein radioactivity. This was chromatographed on a column (20cm x 1.5cm) of Sephadex G15 equilibrated and eluted with 100mM-pyrophosphate buffer, pH 7.4 (Figure 45).

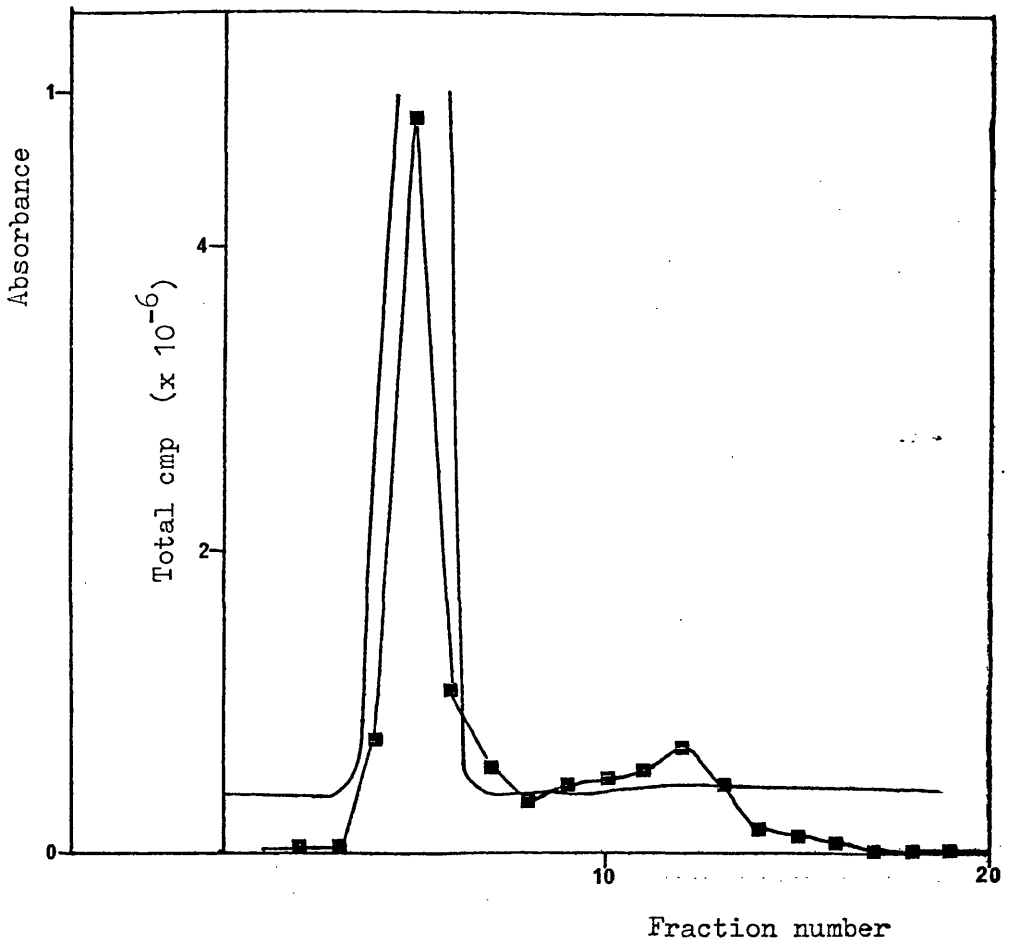
Absorbance at 280nm and radioactivity in the fractions (2.8ml) collected were determined as before. The excluded protein fractions (4-7) contained a total of 1.06×10^6cpm. being 78% of the applied activity (11.5% of total protein radioactivity). The included fractions (8-15) contained 2.46×10^5cpm. (17% of applied activity; 2.66% of total protein radioactivity). This 2.66% of the total original protein radioactivity again represents resolved coenzyme derivative. Thus a total of not more than 4.21% of the coenzyme derivative is resolvable from the enzyme.

It had been hoped that synthetic samples of the

Figure 45

Rechromatography of resuspended protein on Sephadex G15.

The aspartate aminotransferase protein resuspended as described in the text (1ml) was applied to a column (20cm x 1.5cm) of Sephadex G15 equilibrated and eluted with 100mM-pyrophosphate buffer, pH 7.4. Radioactivity (—■—) and absorbance at 280nm (——) of the eluant were determined as described in the text.


Absorbance
1
0
Total cmp (x 10^{-6})
4
2
10
20
Fraction number

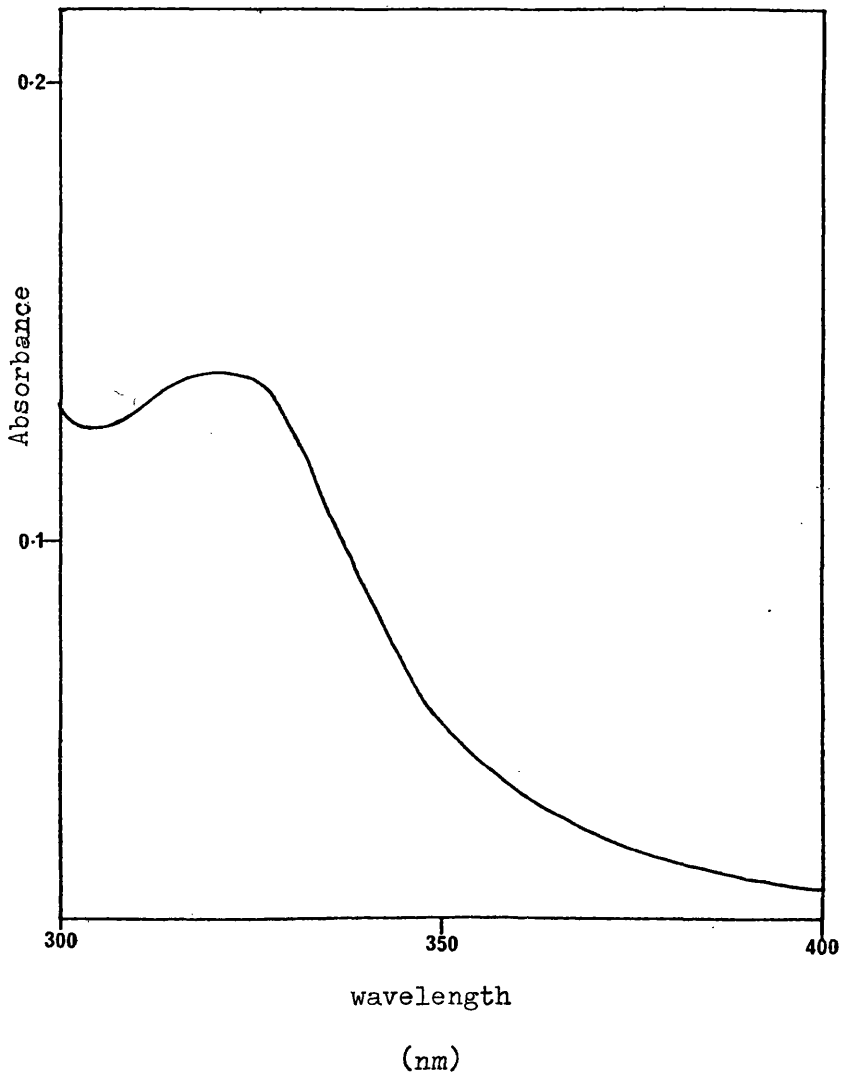
expected resolved reduction product (N(pyridoxal)-difluoro-aspartate) of a Schiff base between enzyme bound pyridoxal-5'-phosphate and difluoro-aspartate could be prepared for the electrophoretic comparison with the resolved radioactivity. However it was not found possible to prepare this compound by the borohydride reduction of any Schiff base formed between pyridoxamine and difluoro-oxaloacetic acid in methanol.

The resolved radioactive fractions ie. the acetone supernatant and the included volumes of Figure 45, were combined after removal of solvent. The absorption spectrum of this fraction was taken by dissolving the residue in 100mM-pyrophosphate buffer, pH 7.4 (2ml) and is shown in Figure 46. The absorption maximum found at ca 330nm is typical of reduced Schiff bases (Severin et al., 1969).

With the assumption that this absorption is due to resolved pyridoxyl-difluoro-aspartate derviative and that this derivative has the same extinction coefficient as that exhibited by the borohydride reduced AAT it may be shown that this absorption can account for 85% of the resolved radioactivity.

Figure 46

Absorption spectrum of the resolved radioactivity.


0·2
0·1
Absorbance
300
350
400
wavelength
(nm)

(6) Circular Dichroism Study of the Binding of Difluoro-aspartate to Aspartate Aminotransferase.

Changes in the induced optical activity of the coenzyme chromophore of AAT on the binding of substrates and pseudo-substrates have provided valuable mechanistic information. This method has also been used to distinguish derviatives of the coemzyme with otherwise identical absorption spectra (Braunstein, 1973). Observations on the optical activity of the difluoro-aspartate AAT complex were therefore undertaken.

(6) i). Changes in the circular dichroism of aspartate amino-transferase on the binding of difluoro-aspartate at pH 7.4.

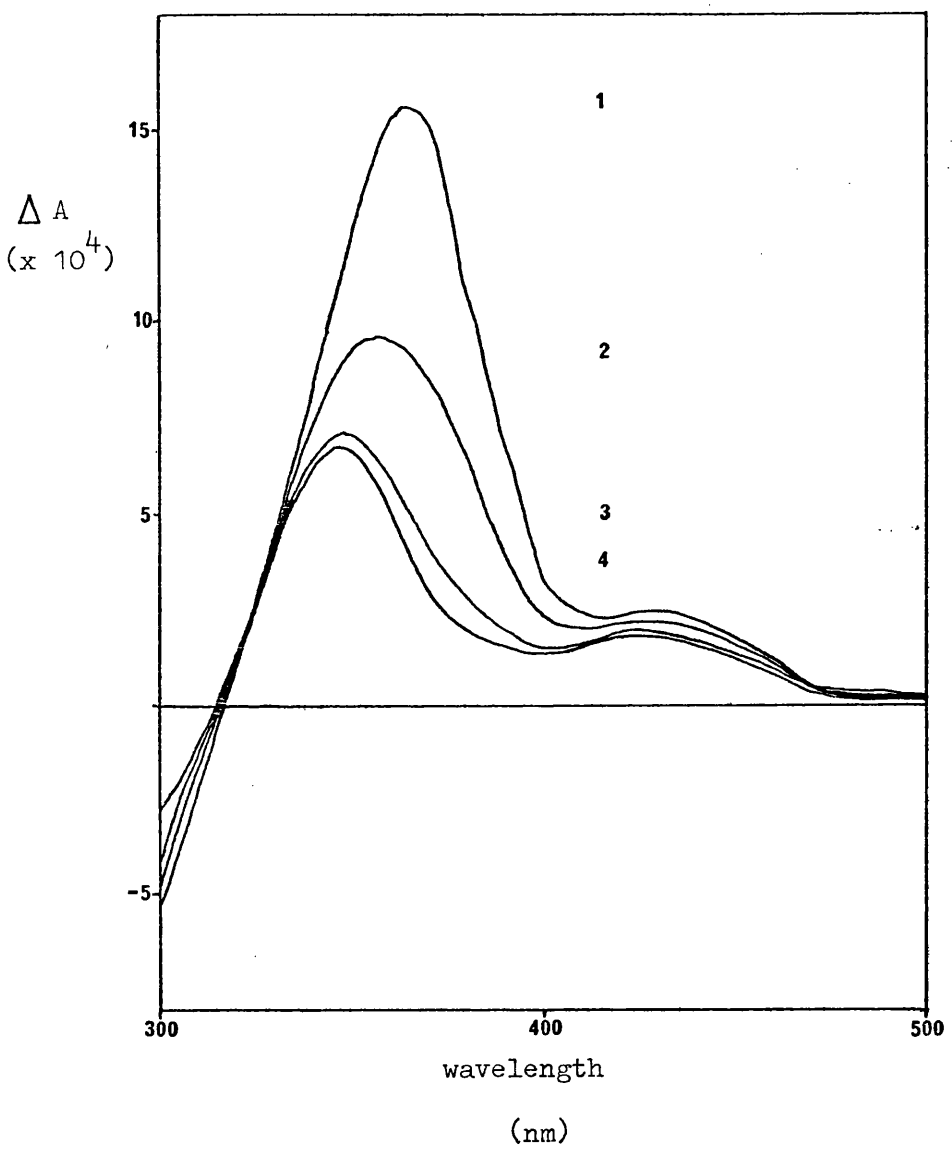
The circular dichroism spectrum of a solution (2.65ml) of the aldimine form of AAT (α-subform; 98.7 μM sites) in 100mM-pyrophosphate buffer, pH 7.4 contained in a 1cm cell was recorded from 500-300nm at a scan rate of 10nm/m in a JASCO J40-CS dichrograph at room temperature. Aliquots (up to a total of 70 μl) of 0.5M-difluoro-aspartate solution were added and the spectra were recorded.

The addition of difluoro-aspartate to AAT resulted in an instantaneous loss in the circular dichroism at 365nm that is associated with coenzyme absorption at 363nm ultimately leaving a new dichroic maximum at 345nm (Fig. 47).

Figure 47

Optical activity changes upon addition of difluoro-aspartate to aspartate aminotransferase at pH 7.4.

1). Circular dichroism spectrum of AAT (α-subform. 98.7 μM in 100mM-pyrophosphate buffer, pH 7.4) and in the presence of 2). 1.88mM; 3). 7.43mM; 4). 12.86mM difluoro-aspartate. The spectra were corrected for dilution on addition of difluoro-aspartate solution.


Δ A
(x 10^4)
15
10
5
-5
1
2
3
4
300
400
500
wavelength
(nm)

(6) ii). Computation of the circular dichroism spectrum for the aspartate-aminotransferase-difluoro-aspartate complex at pH 7.4.

The circular dichroism titration data obtained as above may be interpreted under the same scheme and with the same assumptions as used to interpret the spectral titration data (4 ii)

$$E + S \rightleftharpoons ES$$

where E, aldimine-AAT with a circular dichroism max. at 365nm; S, difluoro-aspartate; ES, enzyme-difluoro-aspartate complex with a circular dichroism max. at 345nm.

The dissociation constant of the complex (ES) is written as;

$$K = [E][S] / [ES] \quad .....(1)$$

If the total concentration of enzyme is Et then

$$[Et] = [E] + [ES] \quad .....(2)$$

If the observed difference in absorbance between left and right circularly polarized light exhibited by a solution of AAT is ΔA_o ($\Delta A_o = A_L - A_R$), and ΔA_S is the same in the presence of difluoro-aspartate then:

$$\Delta A_o = \Delta \varepsilon_E [Et]$$

$$\Delta A_S = \Delta \varepsilon_{ES} [ES] + \Delta \varepsilon_E [E]$$

where $\Delta \varepsilon_E$ and $\Delta \varepsilon_{ES}$ are the differences in extinction coefficient between left and right circularly polarized light for free enzyme and enzyme-difluoro-aspartate complex respectively ($\Delta \varepsilon = \varepsilon_L - \varepsilon_R$).

The change in circular dichroism on addition of difluoro-aspartate is therefore

$$\Delta A_o - \Delta A_S = \Delta\varepsilon_E [Et] - (\Delta\varepsilon_E [E] + \Delta\varepsilon_{ES} [ES]) \quad .....(3)$$

Equations (1) and (2) can yield the following expressions for [E] and [ES]

$$[E] = \left(\frac{[Et]}{1 + K/[S]}\right) \frac{K}{[S]} \quad .....(4)$$

$$[ES] = \frac{[Et]}{1 + K/[S]} \quad .....(5)$$

Substitution of equation (4) and (5) in (3) gives on rearrangement

$$(\Delta A_o - \Delta A_S) = \frac{[Et] (\Delta\varepsilon_E - \Delta\varepsilon_{ES}) [S]}{K + [S]}$$

or

$$\frac{[S]}{(\Delta A_o - \Delta A_S)} = \frac{K}{[Et] (\Delta\varepsilon_E - \Delta\varepsilon_{ES})} + \frac{[S]}{[Et] (\Delta\varepsilon_E - \Delta\varepsilon_{ES})} \quad .....(6)$$

Equation (6) predicts for all wavelengths where $\Delta\varepsilon_E$ differs significantly from $\Delta\varepsilon_{ES}$ that plots of $[S]/(\Delta A_o - \Delta A_S)$ versus [S] will be linear. Two such plots for changes in the circular dichroicity at 365nm and 345nm are presented in Figure 48. For these plots the parameters which define the line (ie. $K/[Et](\Delta\varepsilon_E - \Delta\varepsilon_{ES})$ and $1/[Et](\Delta\varepsilon_E - \Delta\varepsilon_{ES})$ were found from direct linear plots of $1/\Delta A_o - \Delta A_S$ against $[S]/(\Delta A_o - \Delta A_S)$ (Cornish Bowden and Eisenthal, 1978). Thus K may be determined from the data and values of 2.10mM and 2.18mM were found from the changes at 365 nm and 345nm respectively. 95% confidence

Table 5

Visible circular dichroic data of aspartate aminotransferase and its complex with difluoro-aspartate.

Enzyme status	λ_{nm}	$\Delta\varepsilon$ $M^{-1}.cm^{-1}$	$\frac{\Delta\varepsilon}{\varepsilon} \times 10^4$
α-AAT, pH 7.4	365	15.80	20.20
	431	2.43	14.30
α-AAT-difluoro-aspartate	345	5.81	9.08
complex at pH 7.4	431	1.72	10.30
α-AAT, pH 5.5	431	16.41	26.10
α-AAT-difluoro-aspartate	345	5.46	8.40
complex at pH 5.5	431	0.15	1.20

$\Delta\varepsilon$,Difference in molar extinction coefficient for left and right circularly polarized light, calculated as described in text. $\Delta\varepsilon/\varepsilon$, optical anisotropy factor, where ε is molar extinction coefficient at λ , calculated as described in Methods and section 4 ii.

limits for K can not be obtained due to there only being three data points.

$\Delta\varepsilon_{ES}$ may be obtained from the parameter $1/[Et](\Delta\varepsilon_{E} - \Delta\varepsilon_{ES})$ since $[Et]$ is known (Methods) and $\Delta\varepsilon_{E}$ has the following expression:

$$\Delta\varepsilon_{E} = \frac{\Delta A_{o}}{Et.l}$$

where l is the pathlength (cm). Thus the circular dichroism spectrum of the difluoro-aspartate-AAT complex may be computed from estimation of $\Delta\varepsilon_{ES}$ at 5nm intervals throughout the spectra (Fig. 49). The circular dichroism spectrum of the complex shows a weak (relative to free enzyme) dichroic maximum at 345nm with a minor band at 430nm.

Circular dichroism parameters for AAT and its complex with difluoro-aspartate at wavelengths of interest are presented in Table 5. Included in this Table is the anisotropy ratio ($\Delta\varepsilon/\varepsilon$) exhibited by the various forms of the enzyme. This ratio is a measure of the asymmetry of the coenzyme. ε, the extinction coefficient exhibited by the various forms of AAT, was obtained by spectral titration (Section 4) and as described in Methods.

Figure 48

Circular dichroism titration curve for the binding of difluoro-aspartate to aspartate aminotransferase at pH 7.4.

Plots of $[S]/(\Delta A_O - \Delta A_S)$ against $[S]$ taken from the data of Figure 47 at 365nm (●) and at 345nm (○).

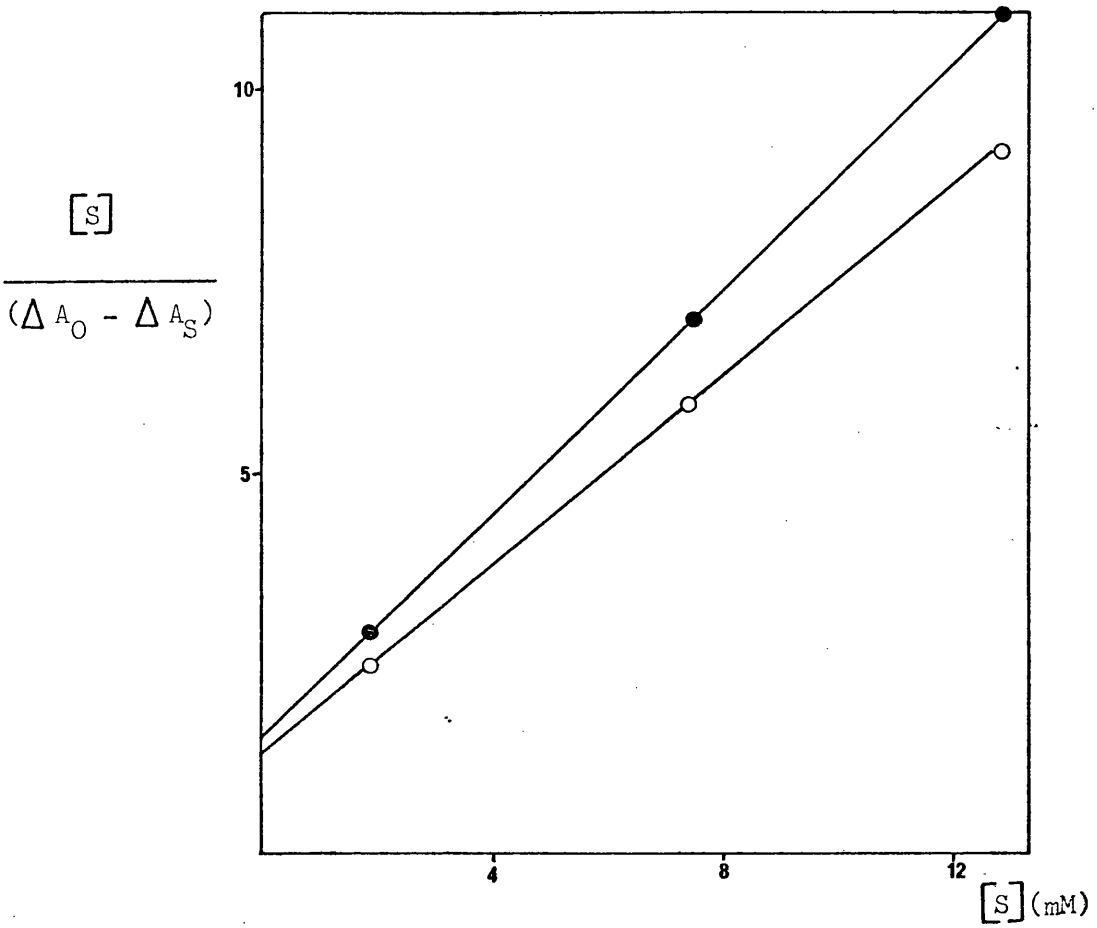
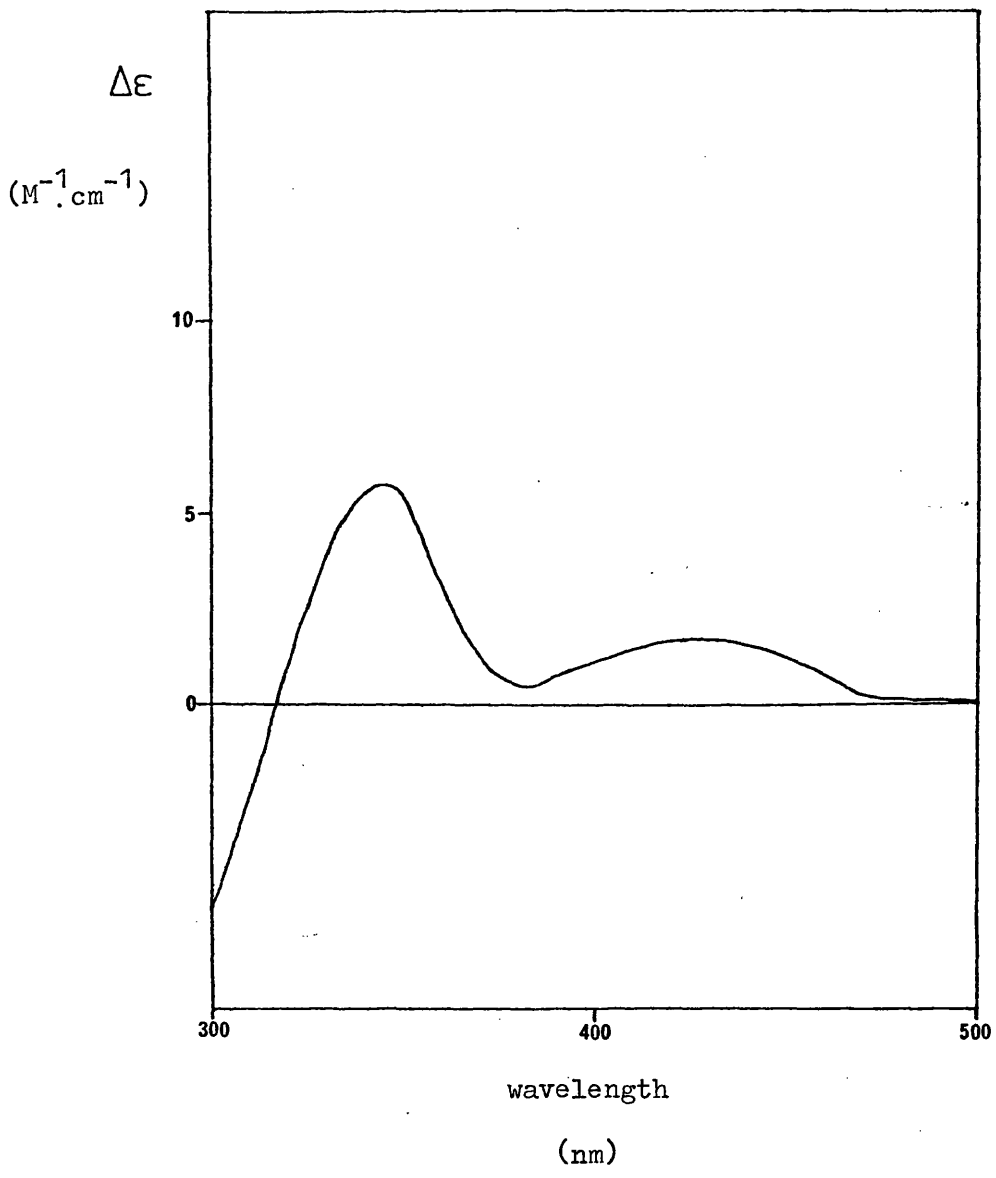

[S]
(Δ A_O - Δ A_S)
10
5
4
8
12
[S] (mM)

Figure 49

Computed circular dichroism spectrum for the aspartate-aminotransferase-difluoro-aspartate complex at pH 7.4.


Δε
(M⁻¹.cm⁻¹)
10
5
0
300
400
500
wavelength
(nm)

(6) iii). Changes in the circular dichroism of aspartate aminotransferase on binding difluoro-aspartate at pH 5.5.

In an identical manner to the study at pH 7.4. α-aldimine-AAT (2.85 ml, 90.8 μM in 100 mM-pyrophosphate buffer, pH 5.5) was titrated with 0.5M-difluoro-aspartate (aliquots to a total of 160 μl) and the circular dichroism spectra recorded (Fig. 50).

This addition of difluoro-aspartate to AAT resulted in an instantaneous loss in the dichroism at 431 nm and the formation of a new weak dichroic maximum at 345nm.

These data may be interpreted under the same scheme as the titration at pH 7.4. Plots of $[S] / \Delta A_o - \Delta A_s$ versus 1/ [S] were linear and gave value for K of 11.0 and 14.75 mM at 430nm and 345nm respectively (Fig. 51). The circular dichroism spectrum of the AAT-difluoro-aspartate complex at pH 5.5 was also computed (Fig. 52). As at pH 7.4 the complex at pH 5.5 has a circular dichroism maximum at 345nm of approximately equal magnitude (Table 5). However the minor absorption peak at ca 430nm and at pH 5.5 (Fig. 39) of the ES complex is practically adichroic. This is in contrast with the same absorption band at pH 7.4 (Fig. 36) which is optically active (Table 5).

Figure 50

Optical activity changes upon addition of difluoro-aspartate to aspartate aminotransferase at pH 5.5.

1). Circular dichroism spectrum of AAT (α-subform; 90.8 μM in 100mM-pyrophosphate buffer, pH 5.5) and in the presence of 2). 5.21mM; 3). 10.32mM; 4). 26.6mM difluoro-aspartate.

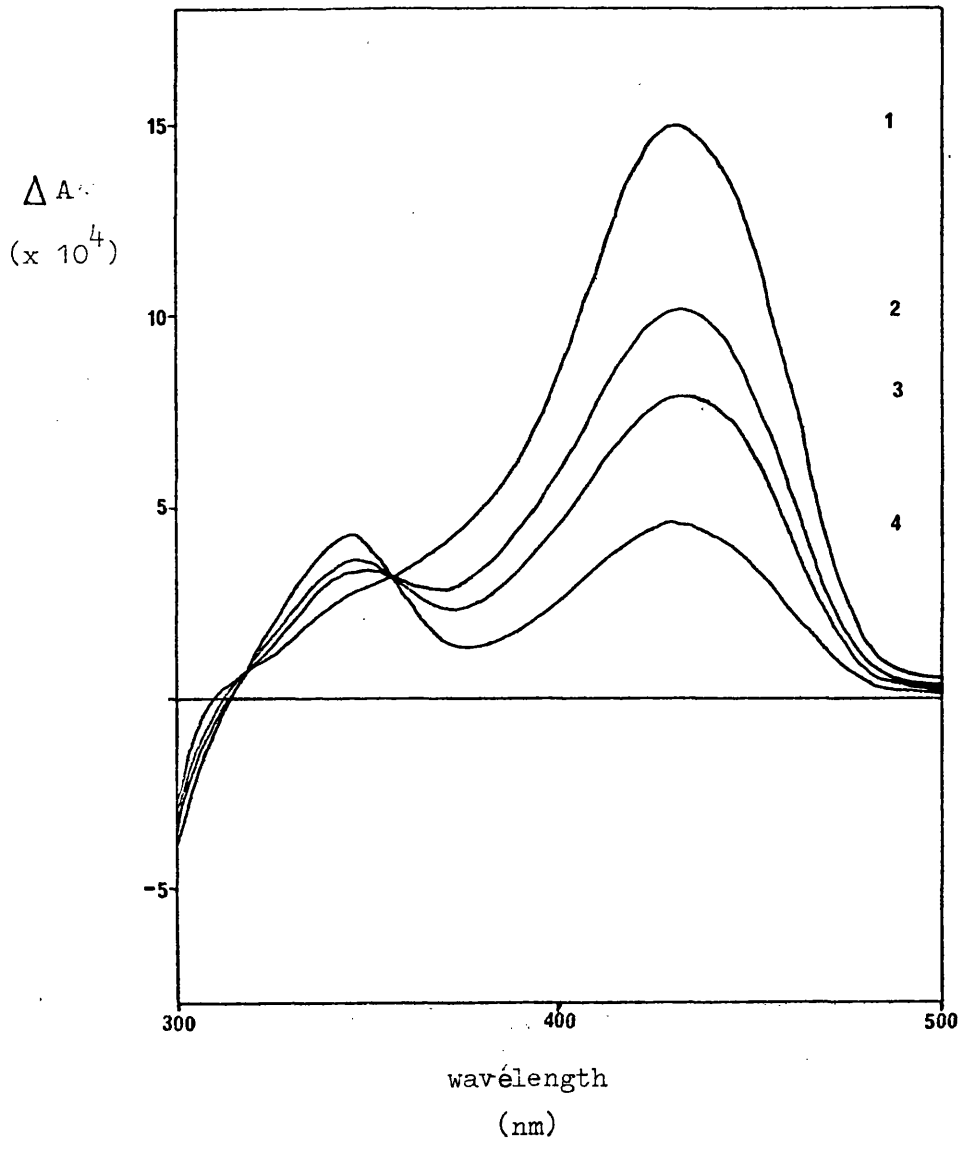

ΔA
(x 10^4)
15
10
5
-5
1
2
3
4
300
400
500
wavelength
(nm)

Figure 51

Circular dichroism titration curve for the binding of difluoro-aspartate to aspartate aminotransferase at pH 5.5.

Plots of $[S]/(\Delta A_O - \Delta A_S)$ against $[S]$ taken from the data of Figure 50 at 431nm (●) and 345nm (○).

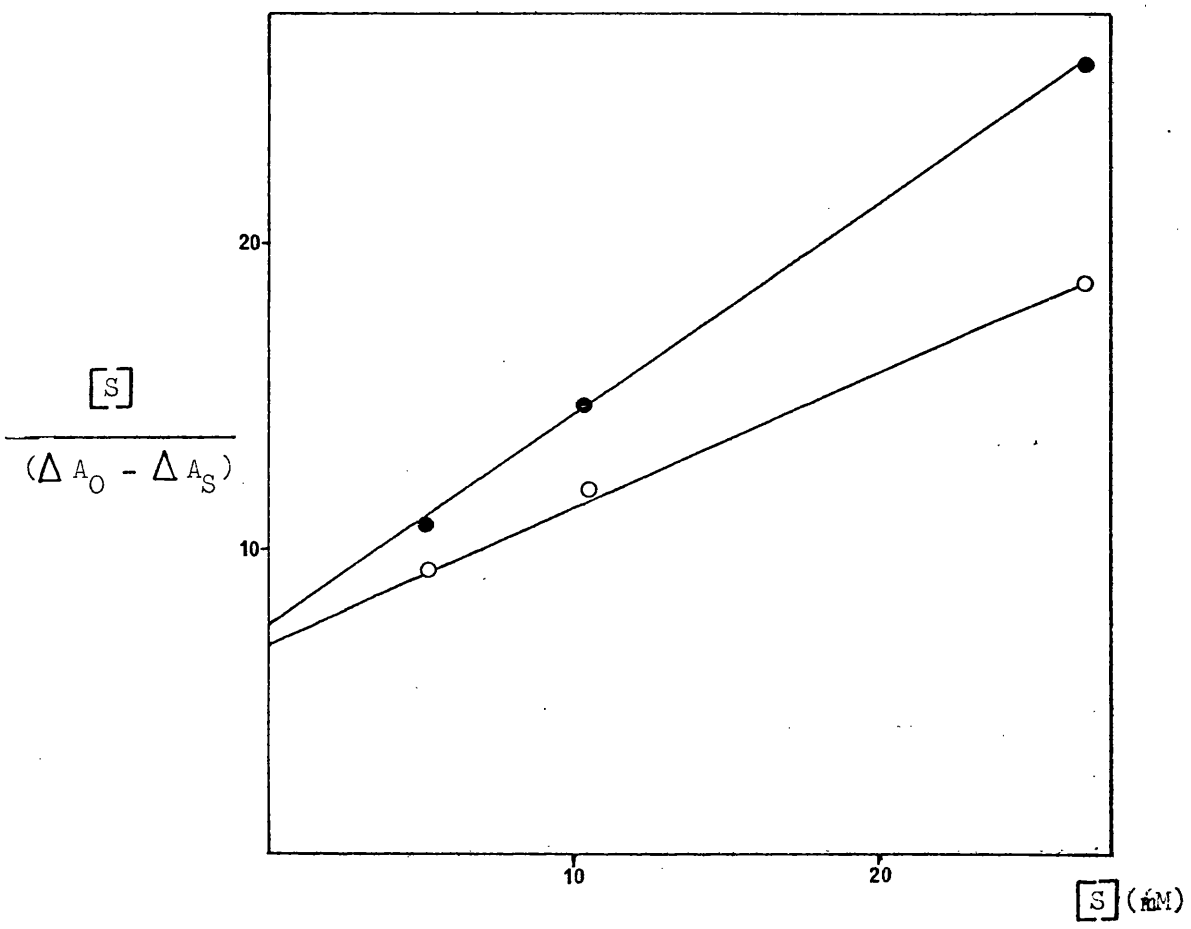
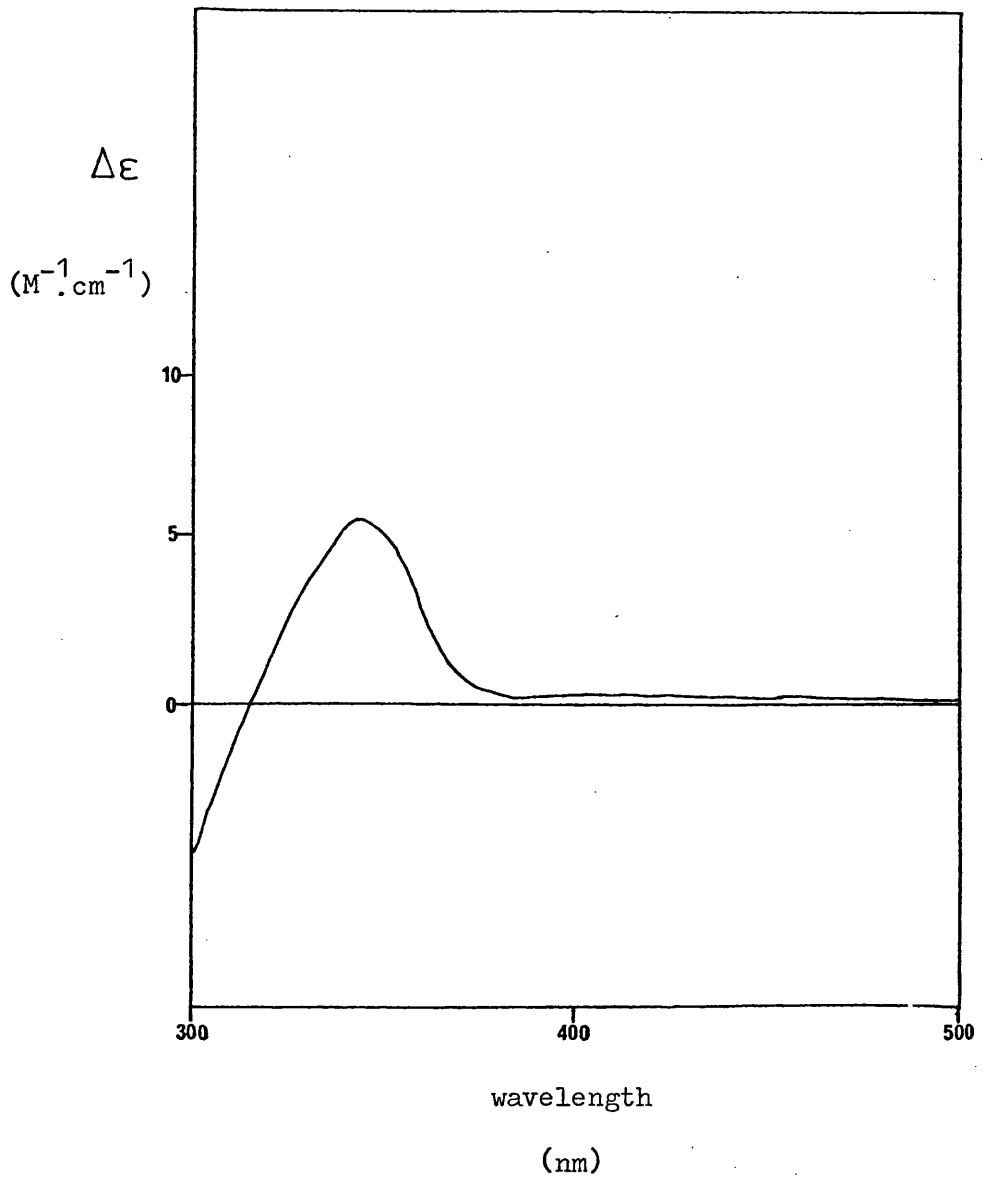

[S]
(ΔA_O - ΔA_S)
20
10
10
20
[S] (μM)

Figure 52

Computed circular dichroism spectrum for the aspartate-aminotransferase-difluoro-aspartate complex at pH 5.5.


Δε
(M⁻¹.cm⁻¹)
10
5
0
300
400
500
wavelength
(nm)

(7) Attempt to Demonstrate Transamination of Difluoro-Aspartate by Aspartate-Aminotransferase.

Interpretation of the data obtained from the studies of an enzyme with a substrate analogue requires caution when one wishes to extrapolate this information to the natural workings of that enzyme. However if the substrate analogue can be shown to be converted to a corresponding product analogue then the validity of the use of the analogue is to some extent established. It was with this in mind that a preliminary study of the substrate activity of difluoro-aspartic acid was undertaken.

(7) i). Spectral changes resulting from prolonged incubation of difluoro-aspartate with the aldimine form of aspartate aminotransferase.

Addition of difluoro-aspartate (to a final concentration of 12.5mM) to a solution of AAT (aldimine form; 46 μM in 100mM-pyrophosphate buffer, pH 7.4) caused a rapid shift in λmax from 360nm to 340nm giving an absorbance spectrum (Fig. 53) which changed only slightly over a period of hours. The major secondary absorbance change was at 318nm but this (Fig. 53a) was only of small amplitude. These events can be described in terms of the reaction scheme shown (Fig. 54 parts (1) and (2) where the slow step $X_1 \longrightarrow X_2$ represents the slow secondary spectral shift. The dissociation constant (K) for the enzyme-difluoro-aspartate complex X_1 was

Figure 53i

Changes in the spectrum of aspartate aminotransferase resulting from prolonged incubation with difluoro-aspartic acid.

1). Spectrum of aldimine AAT (46 μM; in 100mM-pyrophosphate buffer, pH 7.4; 2). as 1) immediately after the addition of difluoro-aspartic acid to a final concentration of 12.5mM;
3). as 2) after a period of 9 hours.

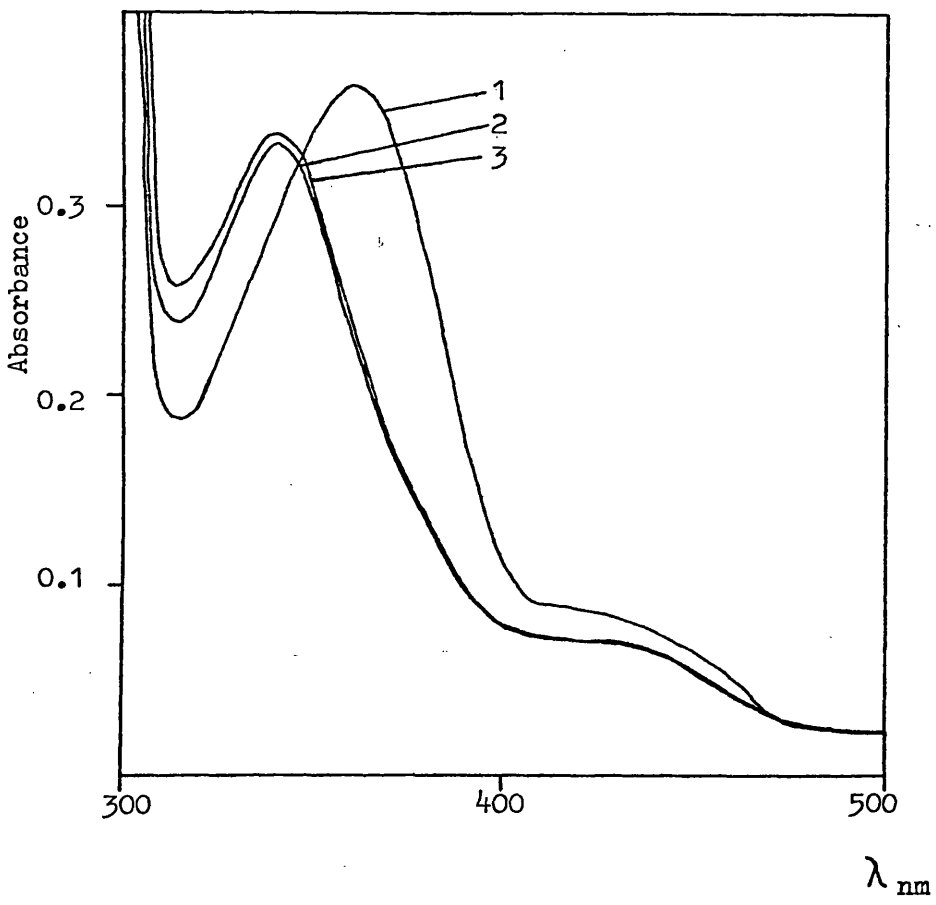
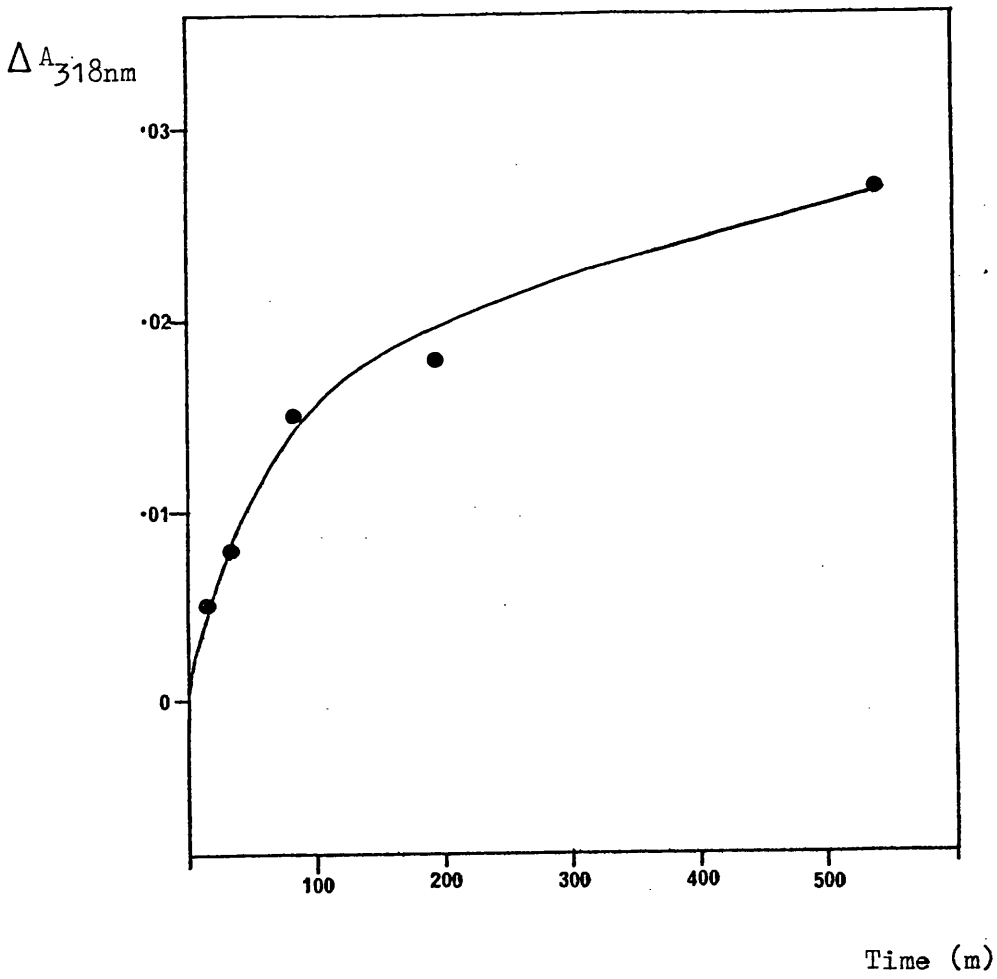

1
2
3
Absorbance
0.3
0.2
0.1
300
400
500
λ nm

Figure 53ii

Changes in absorbance at 318nm of the aspartate-aminotransferase-difluoro-aspartate complex with time.

Plot of absorbance change at 318nm of the AAT-difluoro-aspartate complex with time from the data as obtained in Figure 53, (intermediate spectra in Figure 53i was ommitted for clarity).


ΔA_{318nm}
·03
·02
·01
0
100
200
300
400
500
Time (m)

determined by titration to be 2.5mM.

(7)ii). NADH oxidation in the presence of difluoro-aspartate, aspartate aminotransferase and malate dehydrogenase.

The preceding observations clearly constitute scant evidence for transamination of the substrate analogue and attempts were accordingly made to observe keto acid production by monitoring NADH oxidation in the presence of malate dehydrogenase. The scheme for this is presented in Figure 54, where if P is difluoro-oxaloacetate it is known to be an adequate substrate of malate dehydrogenase (Kun et al., 1963).

The above scheme for the detection of keto acid product is however complicated by the oxidation of NADH that occurs in the presence of AAT alone. This reaction has been studied by Shemisa et al., (1972) and also requires ammonium ions. Thus in a system (1ml; in a 1cm quartz cuvette) comprising 70 μM-AAT (sites), 200 μM-NADH in 100mM-pyrophosphate buffer, pH 7.4 an initial rate of NADH oxidation (observed as change in absorbance at 340nm) of 0.0024 OD units/m is obtained upon the addition of 10 μl of a malate dehydrogenase suspension in 2.2M-$(NH_4)_2SO_4$(2750 i.u./ml). This rate can be reduced to 0.000062 OD units/m by extensive dialysis of both enzymes prior to assay. Under these conditions this 'endogenous rate' is then considerably less than the observed initial rate after the addition of difluoro-

aspartate.

For example with a concentration of 84 μM-AAT, 200 μM-NADH in 100mM-pyrophosphate buffer, pH 7.4 (1ml), with the addition of malate dehydrogenase (27.5i.u.) a rate of NADH oxidation measured at 340nm of 0.097 μM/m was observed and upon the further addition of 25 μl of a 250mM-difluoro-aspartate solution an initial rate of 1.06 μM/m was observed.

Thus with the above system it was possible to demonstrate that after subtraction of the endogenous rate the initial rate of NADH oxidation was dependent on the difluoro-aspartate concentration (Fig.55) and at constant difluoro-aspartate concentration was proportional to the concentration of AAT (Fig. 56).

Progress curves obtained as above were linear for several minutes and the initial linear change in absorbance at 340nm is expressed in μM/m on the assumption that the change in absorbance at 340nm was due only to the oxidation of NADH. The variation of initial rate with difluoro-aspartate may be interpreted under a condensed form of the scheme of Figure 54.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \xrightarrow{k_{cat}} P \qquad \ldots(1)$$

$$P + NADH \xrightarrow[\text{dehydrogenase}]{\text{malate}} NAD + PH_2 \qquad \ldots(2)$$

where E, S, X_1, P, and PH_2 are as defined in Figure 54.

Therefore, as implied by the result of Figure 56, with the reaction (2) not rate limiting, the variation of initial rate with difluoro-aspartate corresponds to the Briggs-Haldane treatment for a single substrate enzyme:-

$$v = \frac{k_{cat}\ [S]}{K_m + [S]}$$

where v is the initial rate in turnover units and

$$K_m = (k_{-1} + k_{cat}) / k_1$$

Thus plots of [S] /v versus [S] are linear (Fig. 57) and afford determination of the Michaelis parameters for the reaction (K_m = 2.5mM; k_{cat} = 2.68 x 10^{-4} s^{-1}). These steady state parameters and hence the line of Figure 57 were determined as described by Cornish-Bowden and Eisenthal (1978).

Similarily at pH 5.5 studies on the reaction of 73 μM-AAT in 100mM-pyrophosphate buffer, pH 5.5 with varying concentrations of difluoro-aspartate yields values for K_m of 8.0mM and k_{cat} = 2.63 x 10^{-4} s^{-1} (Fig.57). However at pH 5.5 difficulty arose with the large background rate (0.395 μM/m) caused by the instability of NADH.

The progress of the reaction of difluoro-aspartate with AAT was monitored as follows. Difluoro-aspartate (final concentration 5mM)was incubated with AAT (final concentration 68.3 μM) in 100mM-pyrophosphate buffer,

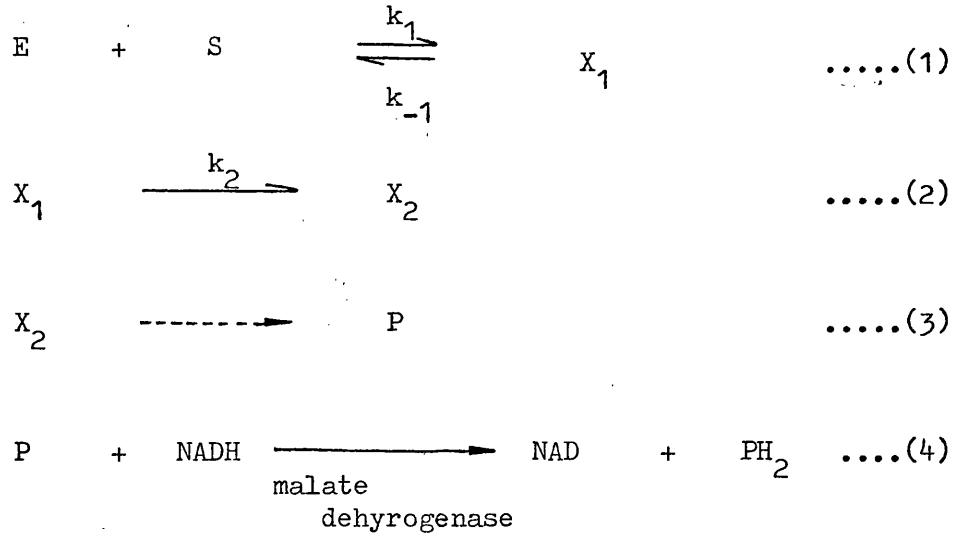
pH 7.4 (5.5ml). At intervals of time an aliquot (1 ml) was removed and assayed for keto acid product. To the sample, in a 1cm quartz cuvette, was added NADH (final concentration 200 μM) upon addition of malate dehydrogenase (27.5 i.u.) the initial rapid decrease in optical density at 340nm is interpreted as the rapid oxidation of accumulated keto acid product (Fig. 58i). The slow rate following this rapid phase is a result of the slow continuing reaction. Therefore it is possible to measure the accumulated keto acid product by the back extrapolation of the slow post rapid phase.

This discontinuous method for following the progress of the reaction was used on account of the possible interference from the 'endogenous' rate.

The time course of keto acid production is presented in Figure 58ii, and was found to follow first order kinetics (Fig. 58iii) with respect to enzyme concentration and with a first order rate constant of 1.005×10^{-4} s^{-1}.

Figure 54

Proposed reaction sequence used to study the substrate activity of difluoro-aspartate with aspartate aminotransferase.



S, difluoro-aspartate; E, aldimine aspartate aminotransferase;

X_1, enzyme difluoro-aspartate complex with λ_{max} 340nm;

X_2, enzyme species resulting from the transformation(s) of X_1;

P, ultimate keto acid product; PH_2, corresponding hydroxy acid.

Figure 55

Variation of initial rate of NADH oxidation with difluoro-aspartate concentration.

Variation of initial rate (v, corrected for 'endogenous' rate by subtraction) of NADH oxidation with difluoro-aspartate concentration [S] for a system (1ml) comprising 84 μM-AAT, 200 μM-NADH, malate dehydrogenase (27.5 i.u.) in 100mM-pyrophosphate buffer, pH 7.4. The reaction was initiated by the addition of difluoro-aspartate after measurement of the 'endogenous rate' (average 0.096 μM/m).

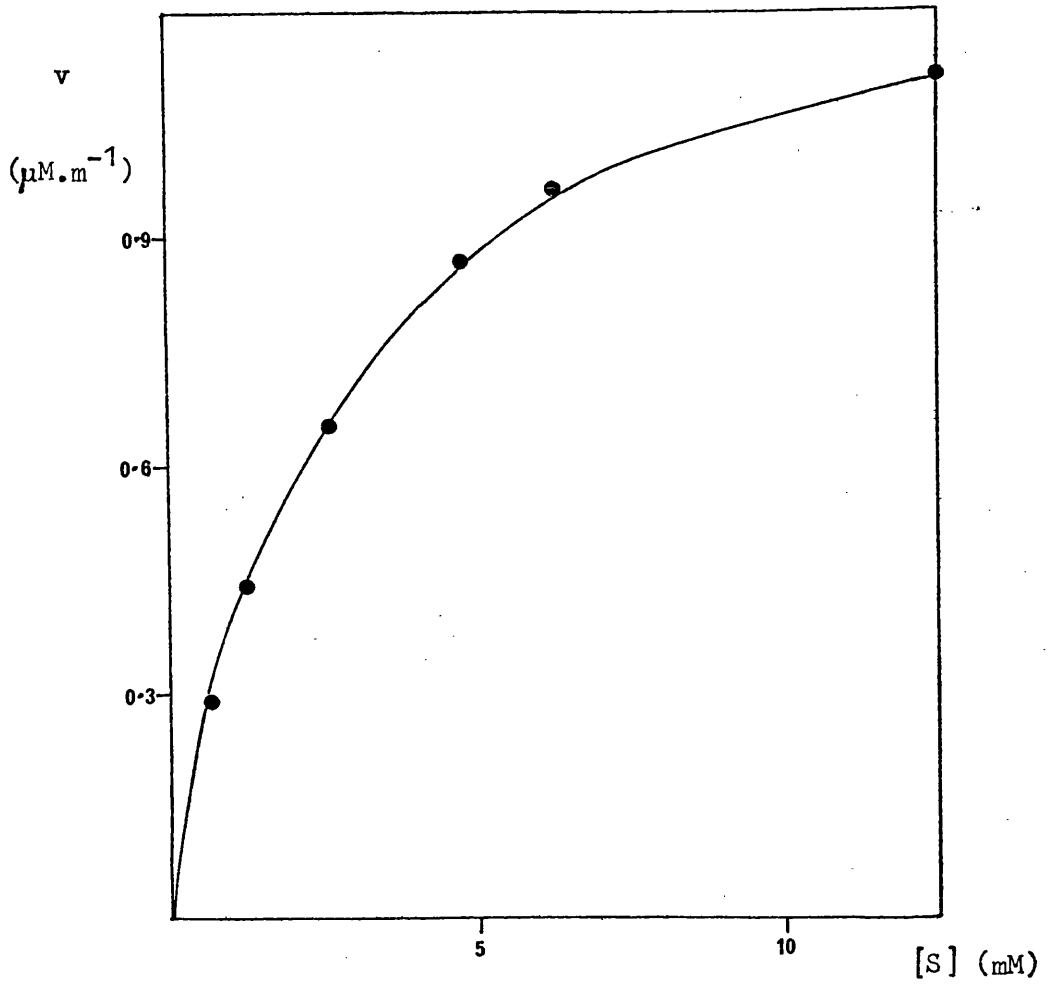

v
(μM.m⁻¹)
0·9
0·6
0·3
5
10
[S] (mM)

Figure 56

Variation of initial rate of NADH oxidation with aspartate aminotransferase concentration.

The conditions of the experiment are as for Figure 55, except that difluoro-aspartate was kept constant (6.25mM) and [AAT] was varied.

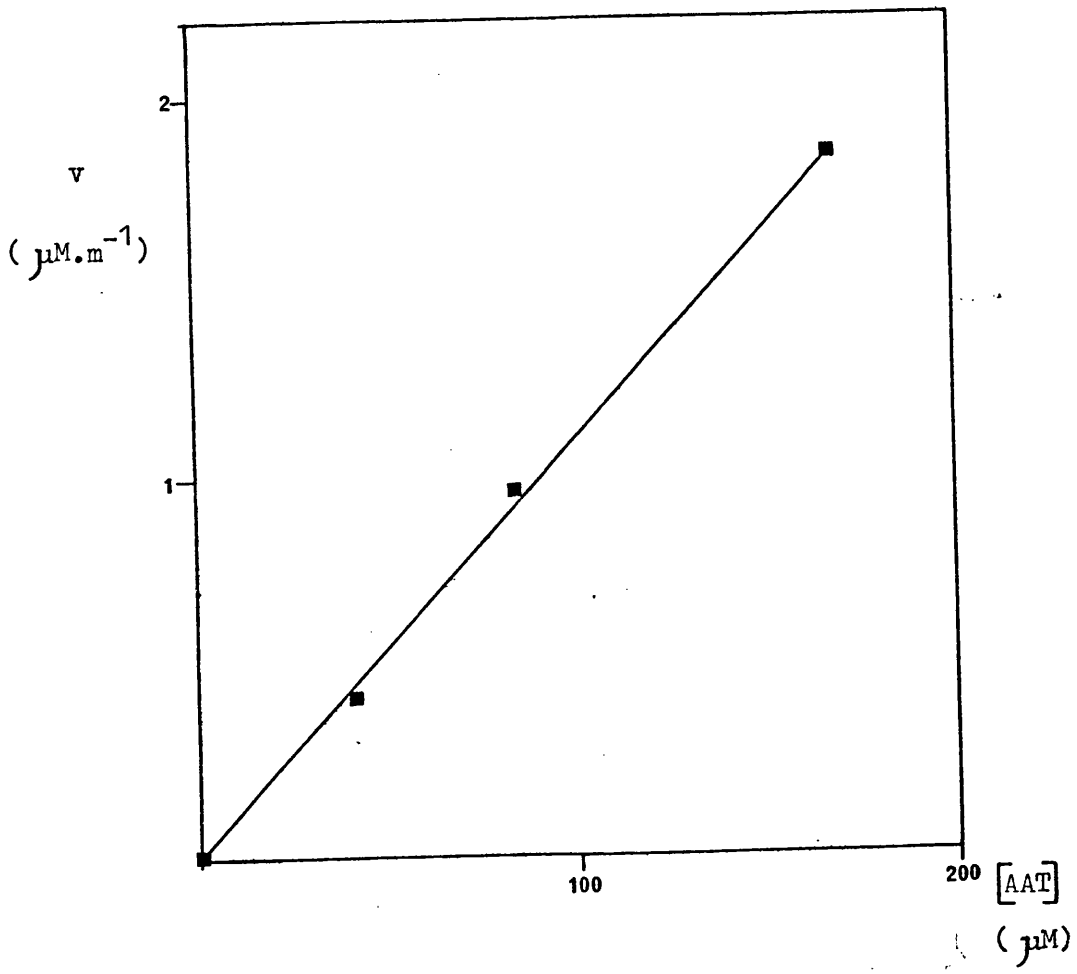

v
(μM.m⁻¹)
2
1
100
200
[AAT]
(μM)

Figure 57

Determination of the Michaelis parameters for the reaction of difluoro-aspartate with aspartate aminotransferase at pH 7.4 and pH 5.5.

Plots of $[S]/v$ against $[S]$ for the reaction of difluoro-aspartate (S) with AAT. The data at pH 7.4 (○) was obtained as described in Figure 55. The data for pH 5.5 (●) was similarly obtained as described in text.

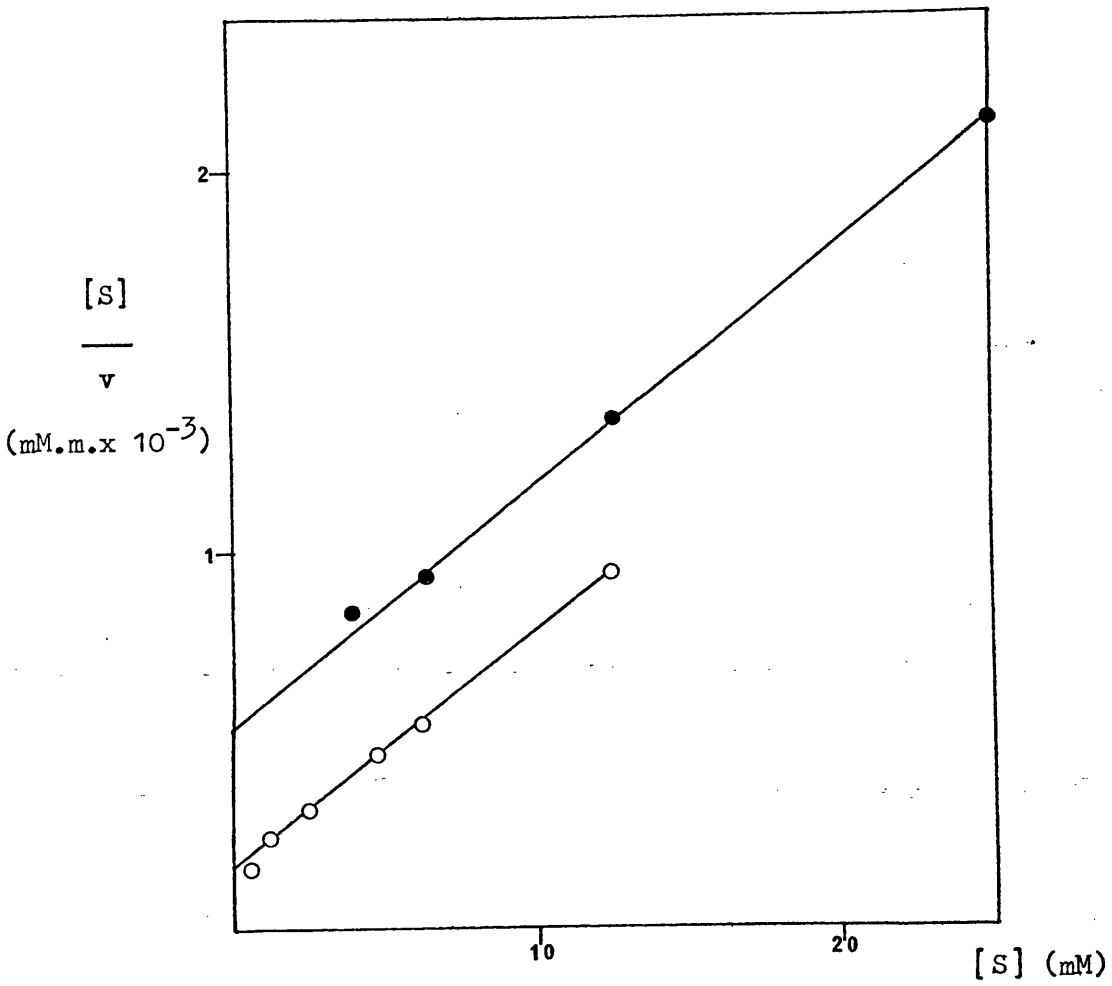

[S]
v
(mM.m.x 10-3)
2
1
10
20
[S] (mM)

Figure 58

Discontinuous assay of the progress of keto acid production on incubation of difluoro-aspartate with aspartate aminotransferase.

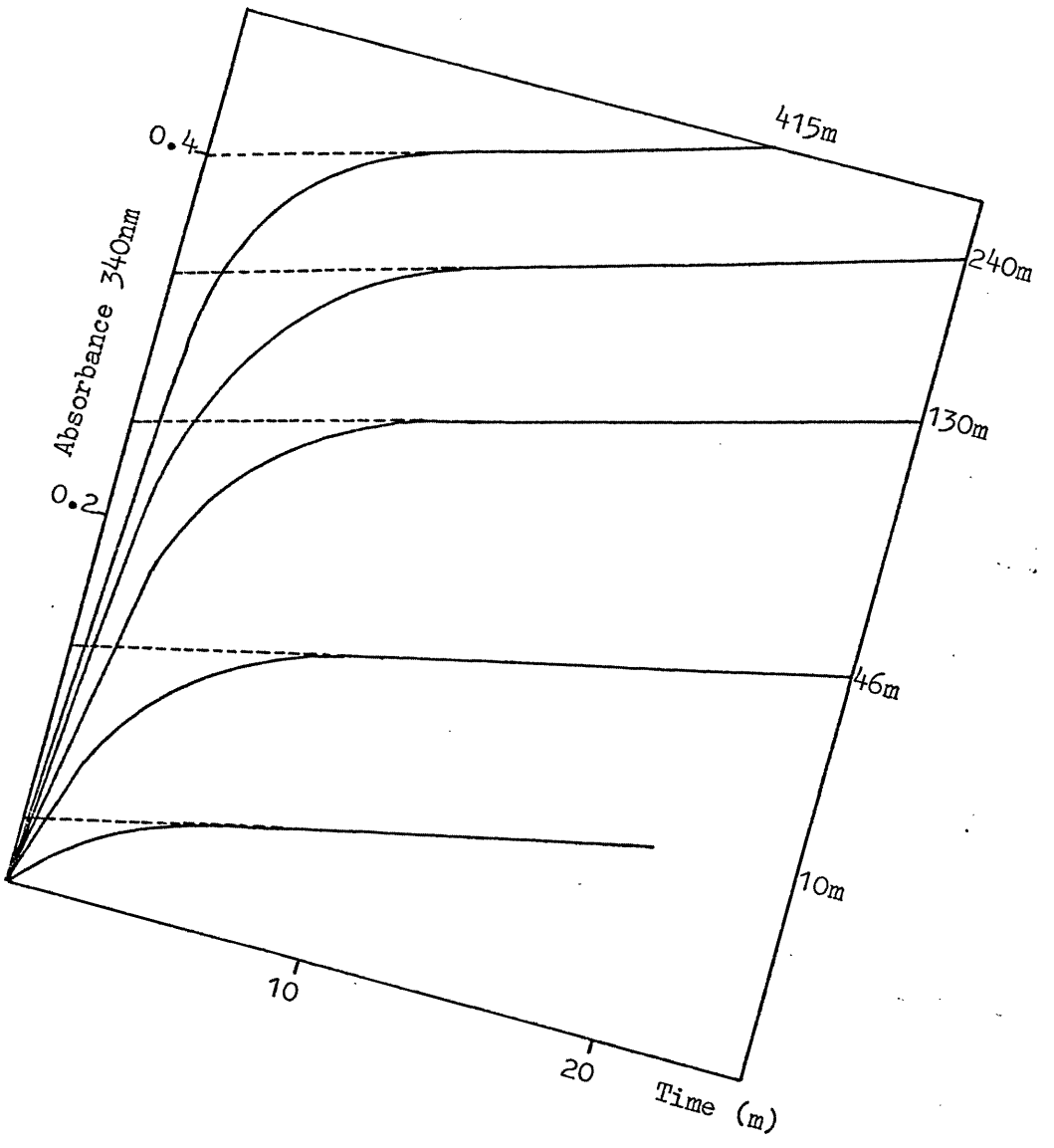
i Progress curves obtained as described in text at the times indicated.

ii Plot of amount of NADH oxidation [x] in the initial rapid phase of the progress curves in (i).

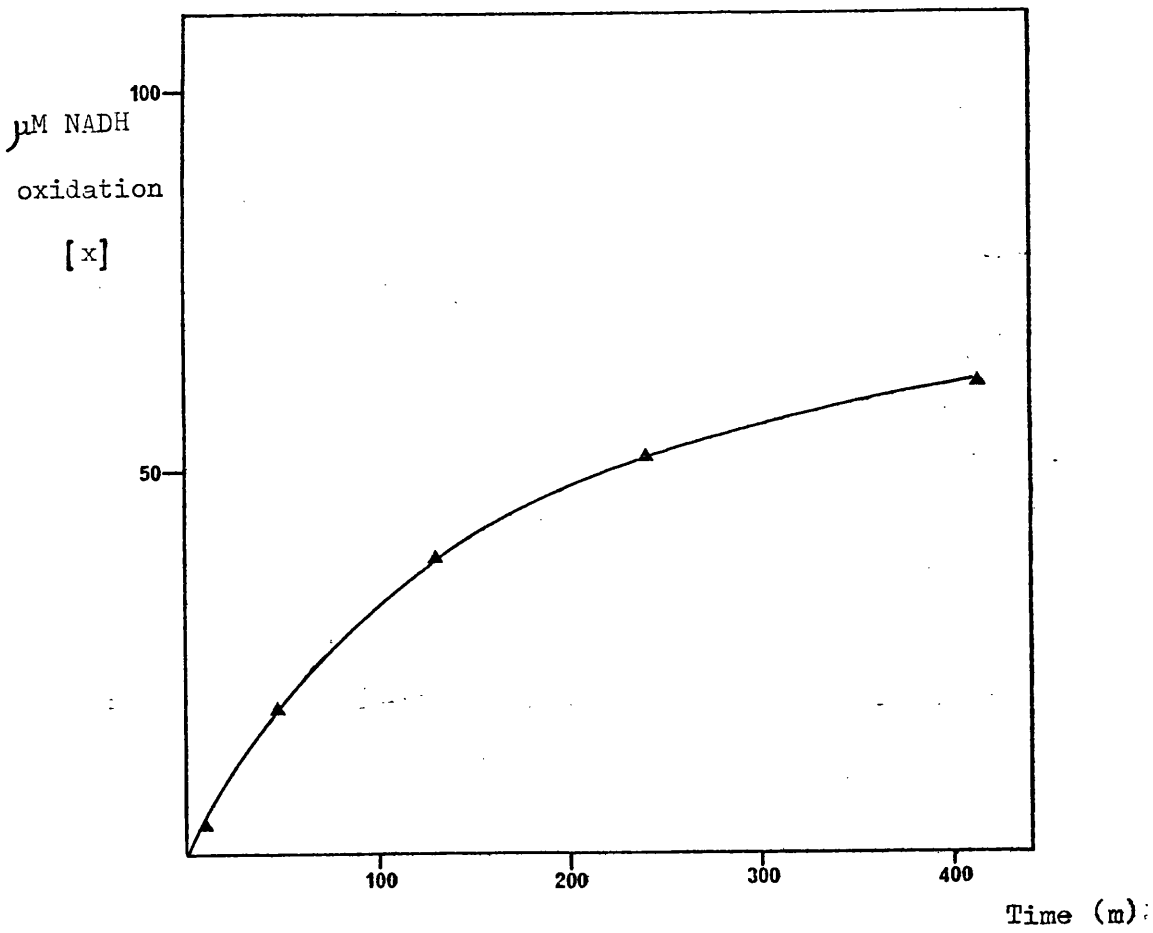
iii First order plot of the data of (ii), total enzyme concentration [Et] = 68.3 uM, details in text.

i

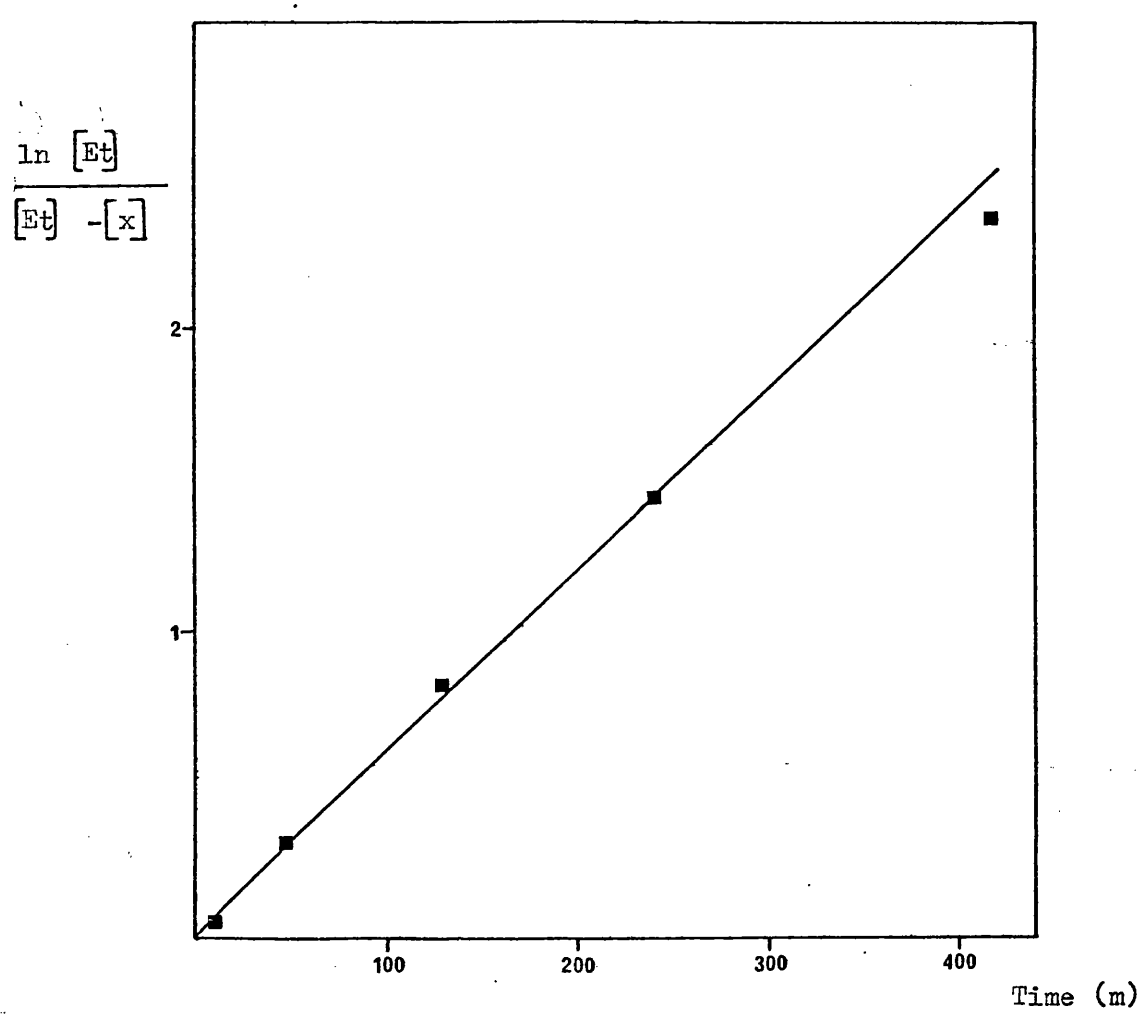
211



ii



iii



(7) iii). Assay of aldimine-aspartate aminotransferase at time intervals following addition of difluoro-aspartate.

If the observed reaction of difluoro-aspartate with aldimine-AAT is according to the scheme:-

$$E + S \rightleftharpoons E' + P$$

(where E and E' are aldimine and aminic-AAT respectively, S and P are difluoro-aspartate and difluoro-oxaloacetate respectively) then an accumulation of the aminic form of AAT would be expected with the concomittant depletion of aldimine enzyme. Attempts were accordingly made to monitor aldimine enzyme depletion. The method, described and justified by Smith (1978), has the following basis.

Cysteine sulphinate reacts rapidly and irreversibly with aldimine-AAT to form aminic-enzyme, SO_2 and pyruvate (Jenkins and D'Ari, 1966a). Measurements of pyruvate (by the observed oxidation of NADH in the presence of lactate dehydrogenase) therefore provide a 'titre' value for aldimine-AAT. Moreover the reaction

$E' + P \longrightarrow E + S$ has been shown to be so slow (relative to the rate of removal of E by cysteine sulphinate) that 'titre' values obtained as above can provide an estimate of aldimine enzyme (and species in rapid equilibrium with aldimine enzyme) in the presence of aminic-enzyme and difluoro-oxaloacetate, according to scheme (2)

$$E + S \rightleftharpoons E' + P$$

$$E + CyS \longrightarrow E' + \text{Pyruvate} + SO_2 \qquad \ldots(2)$$

$$NADH + \text{Pyruvate} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + NAD$$

In the situation depicted by scheme (2) the addition of cysteine sulphinate would cause an initial rapid pyruvate production (measured as NADH oxidation) equivalent to the amount of aldimine enzyme (and species in rapid equilibrium with aldimine enzyme) followed by a slow phase as more aldimine enzyme is formed from E' and P; the response would be biphasic. Thus it is possible to measure aldimine enzyme by back extrapolation of the slow phase to the time of introduction of cysteine sulphinate.

The method was as follows difluoro-aspartate was added to a solution of aldimine-AAT (35 μM sites; 5.5ml) in 100mM-pyrophosphate buffer, pH 7.4 to a final concentration of 6mM.

At intervals of time up to a total of 360m a 1ml aliquot was removed to which was added NADH to a final concentration of 200 μM in a 1cm semi-micro cuvette. The reaction was initiated by the addition of a five fold molar exess of cysteine sulphinate and 0.2 μg-lactate dehydrogenase and followed at 340nm.

The progress curves obtained, presented in Figure 59i, require an explanation.

In the initial stages of the incubation only an initial rapid oxidation of NADH is observed. This is in accord with the 'titre' value being the total concentration

of active enzyme since very little reaction will have taken place. After a period of time the initial rapid phase is followed by a slow phase: yet back extrapolation of the slow phase (by hand and eye) indicates the same amount of aldimine enzyme (Fig. 59ii).

Concurrent with measurements of aldimine enzyme, enzyme activity was monitored in the incubation mix on a suitably diluted sample by standard assay. Enzyme activity was found to be invariant with incubation time (Fig. 59ii).

At the end of the incubation (360m) the concentration of accumulated keto-acid product was measured by using malate dehydrogenase and NADH as described previously and found to be 32 μM.

Figure 59i

Progress curves obtained upon assay of aldimine-AAT at time intervals following addition of difluoro-aspartate.

The progress curves were obtained as described in text at the time intervals indicated (t =) and also illustrate the 'titre' values obtained by back extrapolation (----), details in text.

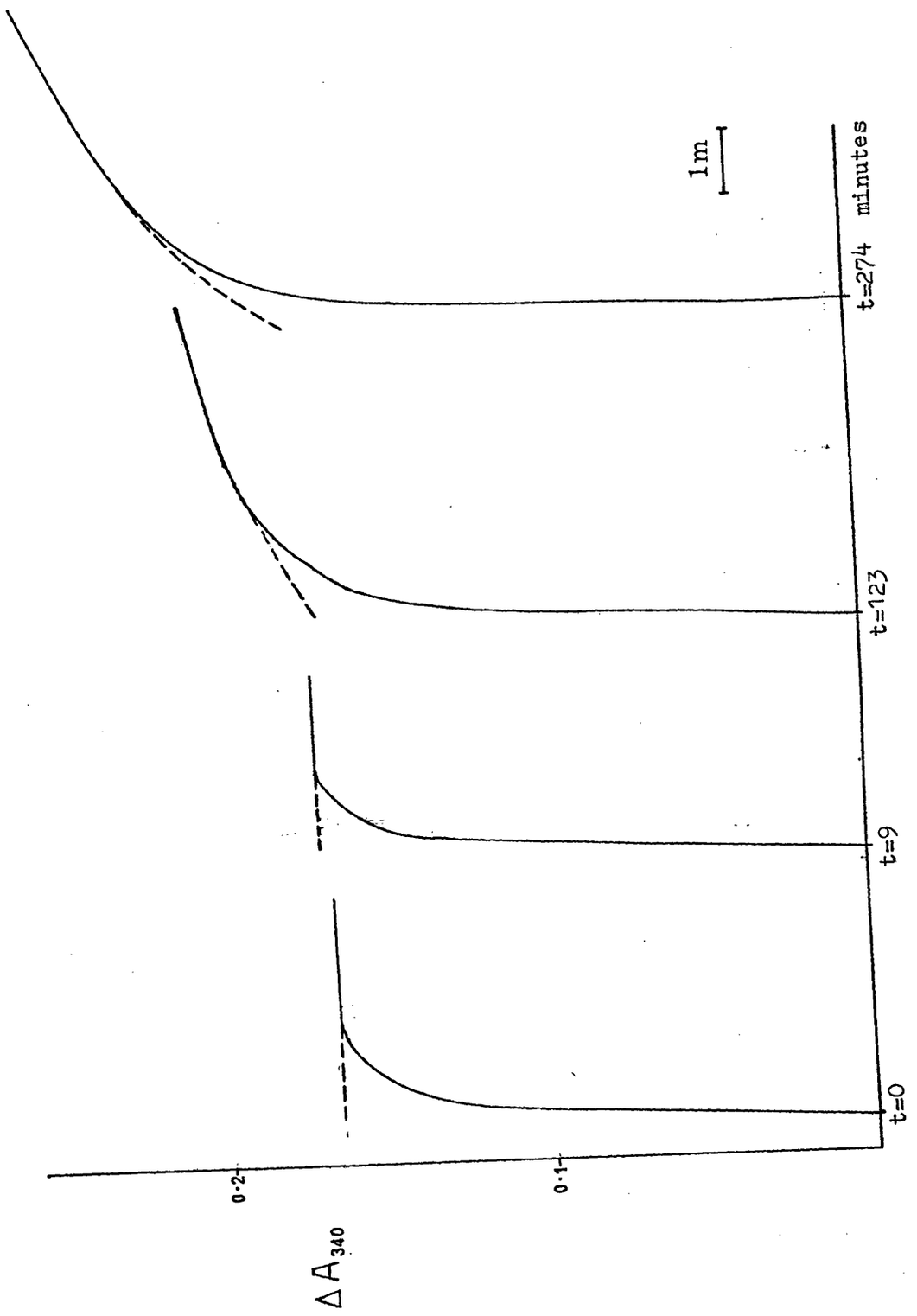
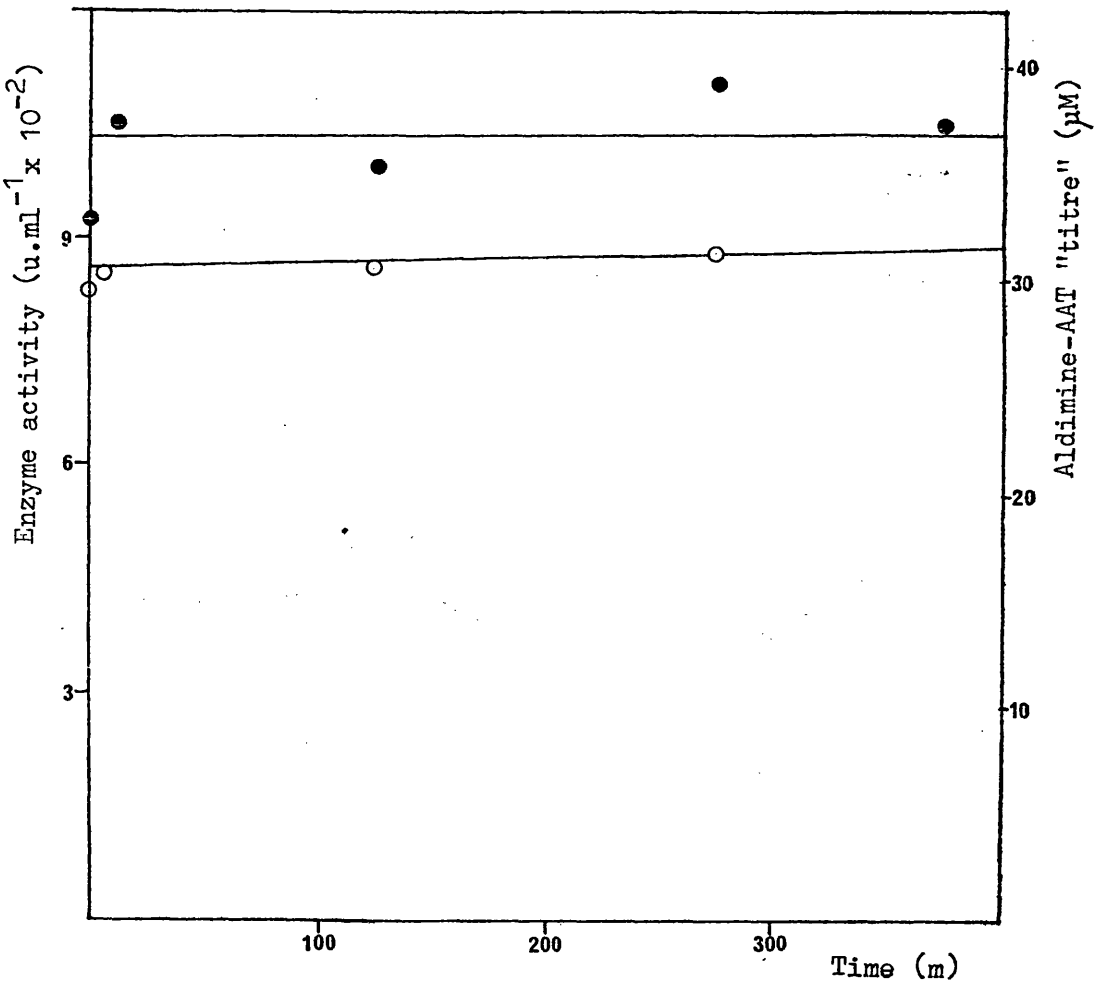

1m
t=274 minutes
t=123
t=9
t=0
0·2
0·1
ΔA340

Figure 59ii

Assay of aldimine-AAT and enzyme activity at time intervals following the addition of difluoro-aspartate.

Plot of 'titre' values for aldimine-enzyme (O) and enzyme activity (●) versus time for an incubation of aldimine-AAT and difluoro-aspartate, details in text.


Enzyme activity (u.ml^{-1} x 10^{-2})
9
6
3
100
200
300
Time (m)
40
30
20
10
Aldimine-AAT "titre" (µM)

Discussion: Part 2

L-Difluoro-aspartic acid.

The enzymic synthesis of this hitherto unknown isosteric difluoro-analogue of aspartic acid was made possible by the observation (Briley et al., 1977a) that the incubation of difluoro-oxaloacetate with AAT and an amino group donor yielded in the n.m.r. spectrophotometer an ^{19}F multiplet corresponding to that expected from difluoro-aspartic acid.

Difluoro-aspartate was accordingly prepared by transamination of difluoro-oxaloacetate with AAT and alanine. Alanine was chosen as the amino group donor because although it is relatively weak substrate for AAT (Jenkins, 1961a) its turnover is some seven times that of difluoro-oxaloacetate (Briley et al., 1977c) and its product, pyruvate, may be simply determined in the presence of difluoro-oxaloacetate so allowing quantification of the reaction.

An essential feature of the method was the slow addition of difluoro-oxaloacetate to the reaction mixture, at a rate equal to the theoretical consumption of the reagent. This was in order to maintain the concentration of difluoro-oxaloacetate below ca 100μM, the concentration at which appreciable quantities of the inhibitory 'abortive' keto-acid-aldimine enzyme complex would be formed (Briley et al., 1977b). The rate of consumption of difluoro-oxaloacetate was computed from the known maximum velocity of the reaction

[1.30×10^{-3} s^{-1} (turnover units) (Briley et al., 1977c)]. The slowing down of the reaction towards the end of the incubation is presumably primarily caused by accumulation of inhibitory concentrations of difluoro-oxaloacetate.

The high yield of purified difluoro-aspartate (typically greater than 70% on the basis of total pyruvate released) provides further support to the conclusion (Briley et al., 1977a) that on reaction of difluoro-oxaloacetate with aminic-AAT an insignificant amount of any β-elimination reaction takes place.

The characteristic ^{19}F n.m.r. spectrum of difluoro-aspartate was used to locate this product and is typical of the A B portion of an ABX system (Jackman, 1959). The two fluorine atoms of difluoro-aspartate are diastereomeric and will therefore exhibit spin-spin coupling; the resulting quartet will in turn be split by the α-proton giving rise to the observed octet.

The optically-active product amino-acid has been assigned as the L-isomer without stereochemical identification. This is a very reasonable assumption because the absolute stereo-specificity of AAT for L-amino-acids has never been impugned. All keto-acids that are transaminated by AAT have been found to be transformed into L-amino-acids (Braunstein, 1973). Furthermore D-amino-acids have not been shown to react with aldimine-AAT. For example in a study (Melander, 1975)

specifically designed to detect any interaction between aldimine-AAT and D-α-methylaspartate none was found.

Some discussion on the properties of difluoro-aspartate is pertinent. As fluorine is the most electronegative element, its presence in a molecule introduces a powerful negative inductive effect which is shown in this case by the effect on the amino-group of difluoro-aspartate. The amino-group of difluoro-aspartate (pK 7.18) is considerably more acidic than that of aspartic acid (pK 9.82). This change in pK is in accord with that found for other fluoro-substituted amino-acids (Loncrini and Filler, 1970).

Such inductive effects and possibly also long range inductive effects will have to be born in mind when considering the covalent congress of AAT and difluoro-aspartate.

According to Kun and Dummel (1969) the presence of an electron donating group (eg. an amino-group) vicinal to a fluorine atom introduces instability and favours HF elimination. This was said to account for the extreme instability of mono-fluoro-aspartate (Kun et al., 1960) which is presumed to exist only as an enzymic intermediate.

Attempts to recrystallize difluoro-aspartate from ethanol-water mixtures at ca 50°C yielded only inorganic

salts, indicating a similar lability of the difluoro-amino-acid. The greater stability of difluoro-aspartate relative to fluoro-aspartate can however be explained by the fact that geminal difluoro-compounds are more resistant to defluorination than mono-fluoro compounds (Kun and Dummel, 1969; Chambers, 1973).

D, L-Difluoro-aspartic acid.

On reflection, failure of all methods designed to prepare difluoro-aspartic acid chemically may be accounted for in the known properties of fluoro-aliphatic acids and it would appear that Kun et al., (1960) had similar difficulty in preparing the mono-fluorinated analogue.

In aqueous solution, difluoro-oxaloacetate probably exists as the hydrate β,β-dihydroxy-difluoro-succinate (Kun and Dummel, 1969) hence failure in the reductive amination of difluoro-oxaloacetate under these conditions may be accounted for by the failure of this compound to react with ammonia to produce the required intermediate imminium moiety (Borch et al., 1971).

In dry methanol, diethyl-difluoro-oxaloacetate exists predominantly as the keto form and upon reductive amination yielded a compound with a proton n.m.r. spectrum attributable to the diethyl-ester of difluoro-aspartate. However the lability of the free acid may

account for the unsucessful attempts at hydrolysis of the ester.

Failure of the reaction of sodium ethyl-phthalimidomalonate with ethyl-chloro-difluoro-acetate can be accounted for by the stabilizing effect of the CF_2 group on the C-Cl bond (Chambers, 1973).

NB

The extrapolations of the progress curves of Figure 59ii are based upon following the curve for a longer period than illustrated in that figure.

The interaction of difluoro-aspartate with aspartate aminotransferase.

The inhibition of the catalytic reaction of AAT by difluoro-aspartate is complex. Difluoro-aspartate acts as a competitive and an uncompetitive inhibitor. According to the analysis of the steady state kinetics difluoro-aspartate is capable of forming an aldimine-enzyme-inhibitor complex as would be expected of a substrate analogue of aspartate. It is also capable of forming an aminic-enzyme-difluoro-aspartate ('abortive') complex and an enzyme-substrate-difluoro-aspartate dead-end complex which would not normally be expected of an analogue of aspartate.

The formation of amino-acid-aminic-enzyme-('abortive') complexes is not apparently paralleled by the natural substrates. Such abortive complexes have not been detected kinetically (Henson and Cleland, 1964) nor have they been detected at equilibrium (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966b). However the substrate analogue erythro- β-hydroxy-aspartate has been shown to interact strongly with pyridoxamine enzyme (Jenkins, 1964). It was suggested that this strong binding and a similar strong binding of meso-tartrate could be explained by the possible presence on the enzyme of group(s) that are capable of binding polar sustitutent in a cis position. A similar argument for the binding of difluoro-aspartate to the aminic-enzyme by virtue of

its polar fluorine atoms can be invoked. Moreover a study of the binding of similar compounds to the aldimine form of AAT again indicated this possibility (Bonsib et al., 1975).

An alternative explanation for the apparently aberrant formation of this 'abortive' complex lies in the predominance of the uncharged amino group of difluoro-aspartate under the conditions used whereas the amino-group of the natural amino acid substrate would be extensively protonated. Binding in the latter form might result in the intrusion of the positively charged amino group into the proximity of the proposed ionized active lysyl residue (Martinez-Carrion et al., 1976; Slebe and Martinez-Carrion, 1976) or the apparently ionized pyridoxamine 4'-amino group (Morino et al., 1978) thus destabilizing the complex in the case of the natural substrates.

There is also an indication that the substrate analogue α- methylaspartate forms an abortive complex with aminic-enzyme (Martinez-Carrion et al., 1973).

The exact nature and mechanism of the formation of the enzyme-substrate-difluoro-aspartate ternary complex proposed to account for the further element of uncompetitive inhibition is not known. The formulation of the mechanism of transamination and its inhibition by substrate analogues generally excludes ternary

complexes of any kind, yet review of the literature has revealed the possibility of such interactions.

The affinity of erythro-β-hydroxy-aspartate for the aminic enzyme is increased in the presence of glutarate or succinate, indicating the formation of ternary complexes (Michuda and Martinez-Carrion, 1970). This was however only observed with the mitochondrial isoenzyme.

In an extensive study of the steady state kinetics of AAT Harruff (1969) discovered the requirement for inhibitory constants of the type K_i'' to explain the inhibition by certain dicarboxylic acids. These constants were however of high value and moderate statistical significance.

As outlined in the Introduction, anomalies in the results of a rapid-reaction kinetic study of the binding of α-methylaspartate to AAT required the proposal of ternary complex formation. In this case however ternary complex formation resulted in activation (Hammes and Haslam, 1968).

Although not, strictly speaking, substrate analogues the anionic bromophenol blue (Harruff and Jenkins, 1976a) and pyrophosphate (Harruff and Jenkins, 1978) form ternary enzyme-substrate-anion complexes.

The results presented cannot be used to ascertain which of the enzyme-substrate-difluoro-aspartate ternary complexes is formed (Fig. 30). An interesting study would be to distinguish the possibilities. Analysis of the effect of difluoro-aspartate on the product inhibition of AAT could provide this distinction, so too possibly could studies on the inhibitory effect of difluoro-aspartate on each half reaction.

Thus the apparently aberrant formation of both an aminic-enzyme-difluoro-aspartate ('abortive') complex and an enzyme-substrate-difluoro-aspartate ternary complex are in accord with some observations by other workers. It may be, however, that formation of such complexes depends upon properties peculiar to the particular substrate analogue chosen for study and, as such, need not necessarily modify our understanding of the mechanism of interaction of AAT with its natural substrate.

The interaction of difluoro-aspartate with the aldimine form of aspartate aminotransferase.

As an analogue of aspartic acid, the interaction of difluoro-aspartate with the aldimine form of AAT is to be expected. Moreover the steady state kinetic estimate of the dissociation constant (K_i) of the aldimine-AAT-difluoro-aspartate complex (K_i = 2.25mM) is comparable to the dissociation constant of the aldimine-AAT-aspartate complex found in temperature jump studies (K = 4.2mM), (Fasella and Hammes, 1967) and from equilibruim spectrophotometric titrations (K = 3.2mM), (Jenkins and Taylor, 1965).

If significant productive breakdown of the aldimine-AAT-difluoro-aspartate complex was occurring under the conditions of the steady-state inhibition experiments the measured K_i would no longer be a good approximation to the true dissociation constant of the above complex. Such a reaction, which would be manifested by a non-hyperbolic variation of the initial rate with respect to aspartate concentration in the presence of difluoro-aspartate, was not detected (Fig. 23-26). This finding is supported by the observation that enzyme concentrations five orders of magnitude greater than those used in the steady-state kinetic experiments were required before de-amination of difluoro-aspartate by AAT was detectable (Section 7ii).

The interaction of difluoro-aspartate with aldimine-

AAT was further studied by spectral titration where it was shown that difluoro-aspartate reacted rapidly with aldimine enzyme to form a complex with a spectral maximum at 340nm and with a minor absorption band *ca* 430nm (Fig. 36).

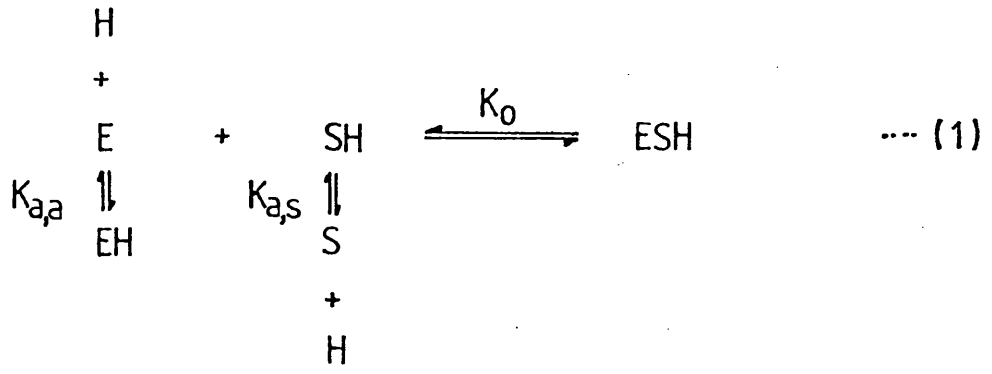
The determined dissociation constant of this complex at pH 7.4 (K = 2.5mM) is in excellent agreement with the steady state kinetic estimate (K_i = 2.25mM) indicating that the complex with λmax 340nm is the species responsible for the competitive inhibition of AAT with respect to aspartate.

The dissociation constant is also in reasonable agreement with a value found previously for a proposed aldimine enzyme-difluoro-aspartate complex (K = 0.7mM) by an indirect method (Smith *et al.*, 1978). This value was obtained as follows: aminic-AAT was permitted to react to completion with difluoro-oxaloacetate; to the completed reaction mixture difluoro-oxaloacetate was added incrementally and the dissociation constant of the 'abortive' aldimine-enzyme-difluoro-oxaloacetate complex was determined by using ^{19}F n.m.r.. The difference of dissociation constant, found as above, from that by direct titration of difluoro-oxaloacetate with aldimine enzyme was used to calculate the dissociation constant of the putative complex of aldimine-AAT and difluoro-aspartate.

Difluoro-aspartate has also been shown to interact with the protonated enzyme that predominates below ca pH 6.5 and absorbs maximally at 430nm to give an enzyme substrate-analogue complex again with an absorption maxima at 340nm and 430nm (Fig. 39). Moreover the molar extinction coefficient at 340nm of the enzyme (α -subform)difluoro-aspartate complex was not significantly different at pH 5.5 and pH 7.4 ($\varepsilon_{340(ES)}$ 6.4×10^3, pH 5.5; $\varepsilon_{340(ES)}$ 6.5×10^3, pH 7.4). The pH invariability of the extinction coefficient at 340 nm ($\varepsilon_{340(ES)}$) of the enzyme-difluoro-aspartate was further demonstrated by a more extensive study using mixed-subform AAT where a weighted mean value for $\varepsilon_{340(ES)}$ of 7.2×10^3 was obtained. The observed difference in $\varepsilon_{340(ES)}$ between the α-subform and the mixed subform complex is in accord with the known presence of enzyme species absorbing maximally at 340nm in mixed subform enzyme (Martinez-Carrion et al., 1967a).

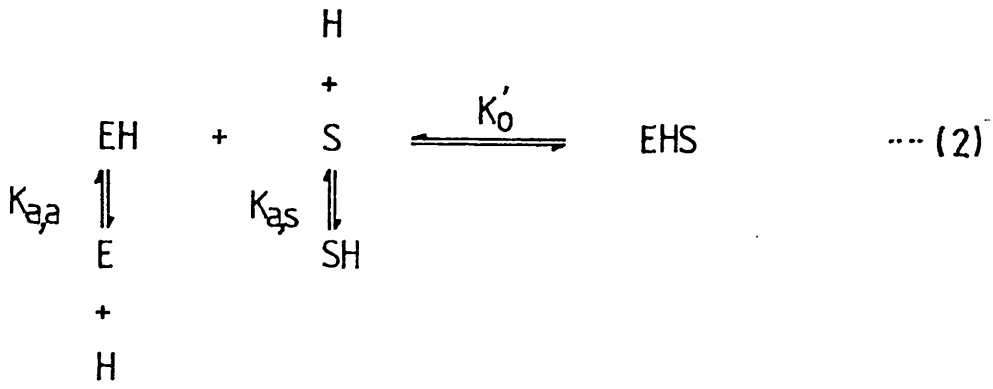
Insensitivity of the spectrum of the enzyme-amino-acid complex to pH has also been observed for the α-methylaspartate (Fasella et al., 1966) and for the erythro- β-hydroxy-aspartate complex (Jenkins, 1964; Harruff and Jenkins, 1978).

In contrast to the invariance of $\varepsilon_{340(ES)}$ the affinity of difluoro-aspartate was however found to vary with pH in a way that was interpreted under and adequately described by the scheme.

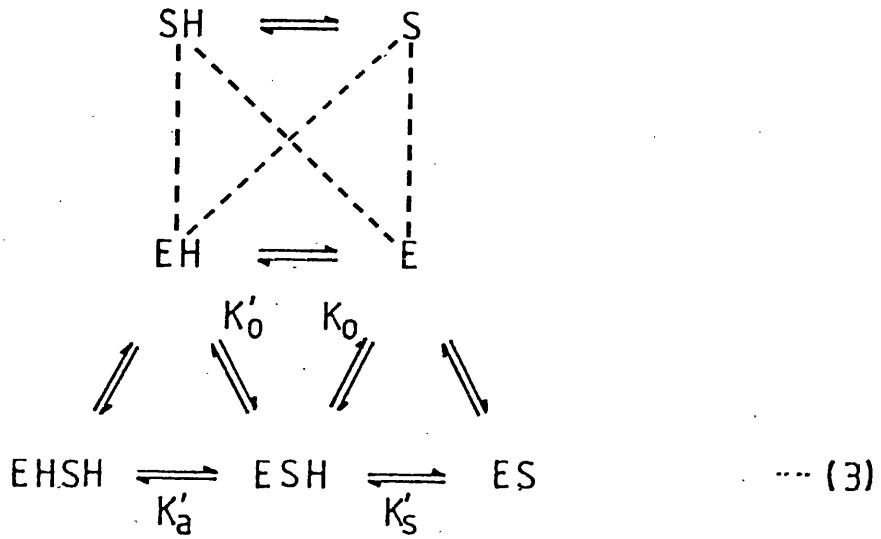


··· (1)

However these data do not allow a distinction between the above scheme and schemes (2) or (3):



··· (2)



··· (3)

where pK_a' and pK_s' are outside the pH range considered. According to schemes (2) and (3) the pH independent dissociation constant (K_o') for the reaction:

$$EH + S \overset{K_o'}{\rightleftharpoons} ESH$$

would be 0.18mM. This value is obtained from the thermodynamic relationship:

$$K_o' = K_o(K_{a,s}/K_{a,a})$$

(where $pK_{a,a} = 6.5$; $K_o = 0.9$mM (Fig. 41ii) and $pK_{a,s} = 7.18$ (Fig. 14)).

In support of schemes (2) and (3) it has been observed that neutral hydroxylamine (presumably acting as a substrate amino-group analogue) reacts preferentially with the protonated enzyme (Velick and Vavra, 1962a, b; Hammes and Fasella, 1963). However the study (Fasella et al., 1966) of the pH variation of the dissociation constant of the α-methylaspartate-AAT complex provided the precedent for scheme (1). Their study which was uncomplicated by substrate ionization provides strong evidence to suggest that unprotonated enzyme reacts with zwitterionic amino-acid.

A more recent interpretation of the pH variation of the dissociation constant of amino-acids and AAT, reported after the completion of the work described in this thesis suggests that it can be explained by the exclusive binding of competitive anions with increasing affinity below ca pH 7.0 (Harruff and Jenkins, 1978), a proposal difficult to reconcile with either scheme (2)

or (3). However these findings can be accommodated in the terms of scheme (1) whereby a value for $pK_{a,a}$ of 6.5 was obtained. This value is consistent with the pK_a of between 6.2 and 6.9 determined for the anion binding site of AAT by Cheng and Martinez-Carrion (1972).

Apparent production of a keto-acid product from AAT and difluoro-aspartate.

It has been shown that on incubation of difluoro-aspartate with the aldimine form of AAT and malate dehydrogenase a slow rate of NADH oxidation was observed and this was interpreted as the formation of a keto-acid product under the relatively open proposals of the scheme of Figure 54.

It is however pertinent initially to discuss other schemes that might explain the results obtained.

For example it is perhaps worth considering further the oxidation of NADH that occurs in the presence of AAT and ammonium ions as outlined by Shemisa et al., (1972). This slow reaction is believed to involve the reductive amination of a keto group on AAT, but is not clearly understood. The interaction of difluoro-aspartate might result in a facilitation of a reaction of this type and therefore also provide results similar to those of Figure 57. Under this proposal the determined Michaelis constant (Km) for the reaction would be comparable with the dissociation constant (K) of the enzyme difluoro-aspartate complex, as was found (cf. pH 7.4, Km = 2.5mM, K = 2.5mM (Fig. 35): pH 5.5, Km = 8.0mM, K = 12.1mM (Fig. 38)). However in the reaction reported by Shemisa et al., up to 15μmole of NADH per μmole transaminase was oxidised which is in contrast to the

1:1 relationship indicated in the present study (Fig. 58iii). Moreover such a proposal would be difficult to reconcile with the observed (Fig. 58i) accumulation of NADH oxidising 'potential' in incubations of difluoro-aspartate and AAT in the absence of NADH (Fig. 58i). Shemisa et al., (1972) also noted that a variety of substrates and substrate analogues have no significant effect on the initial rate of their reaction.

The correspondence of the dissociation constant of the enzyme-difluoro-aspartate complex (K) with the Michaelis constant (Km) for the reaction strongly indicates that the observed reaction is neither catalysed by an impurity nor is a result of non enzymic breakdown of difluoro-aspartate (this was also verified experimentally), and is fully in accord with the scheme presented in which keto acid production is a result of the slow productive breakdown of the enzyme-difluoro-aspartate complex with λ_{max} 340nm.

ie. $$Km \simeq K$$

$$\frac{k_{-1}+k_{cat}}{k_{+1}} \simeq \frac{k_{-1}}{k_{+1}}$$

provided that $k_{cat} \ll k_{+1}[S]$ or k_{-1} which has been shown (cf. pH 7.4, $k_{cat} = 2.68 \times 10^{-4} s^{-1}$; $k_{+1} > 2.5 \times 10^{5} M^{-1}.s^{-1}$, $k_{-1} > 600\ s^{-1}$, (stopped flow)).

The comparability of the turnover of the reaction

found for pH 7.4 and pH 5.5 (k_{cat} = 2.63 x $10^{-4}s^{-1}$) suggests a pH insensitivity for this parameter in the pH range 5.5 - 7.4 and in view of the pH insensitivity of the spectrum of the difluoro-aspartate-AAT complex (Table 4) lends further support to the above conclusion.

The progress of the reaction was followed, where that rate of reaction was shown to decrease with time (Fig. 58ii) by a first order process with respect to enzyme concentration. The first order rate constant found as above (k = 1.005 x $10^{-4}s^{-1}$) is reasonably consistent with the value of k_{cat} found from initial rate studies considering the theorectical relationship

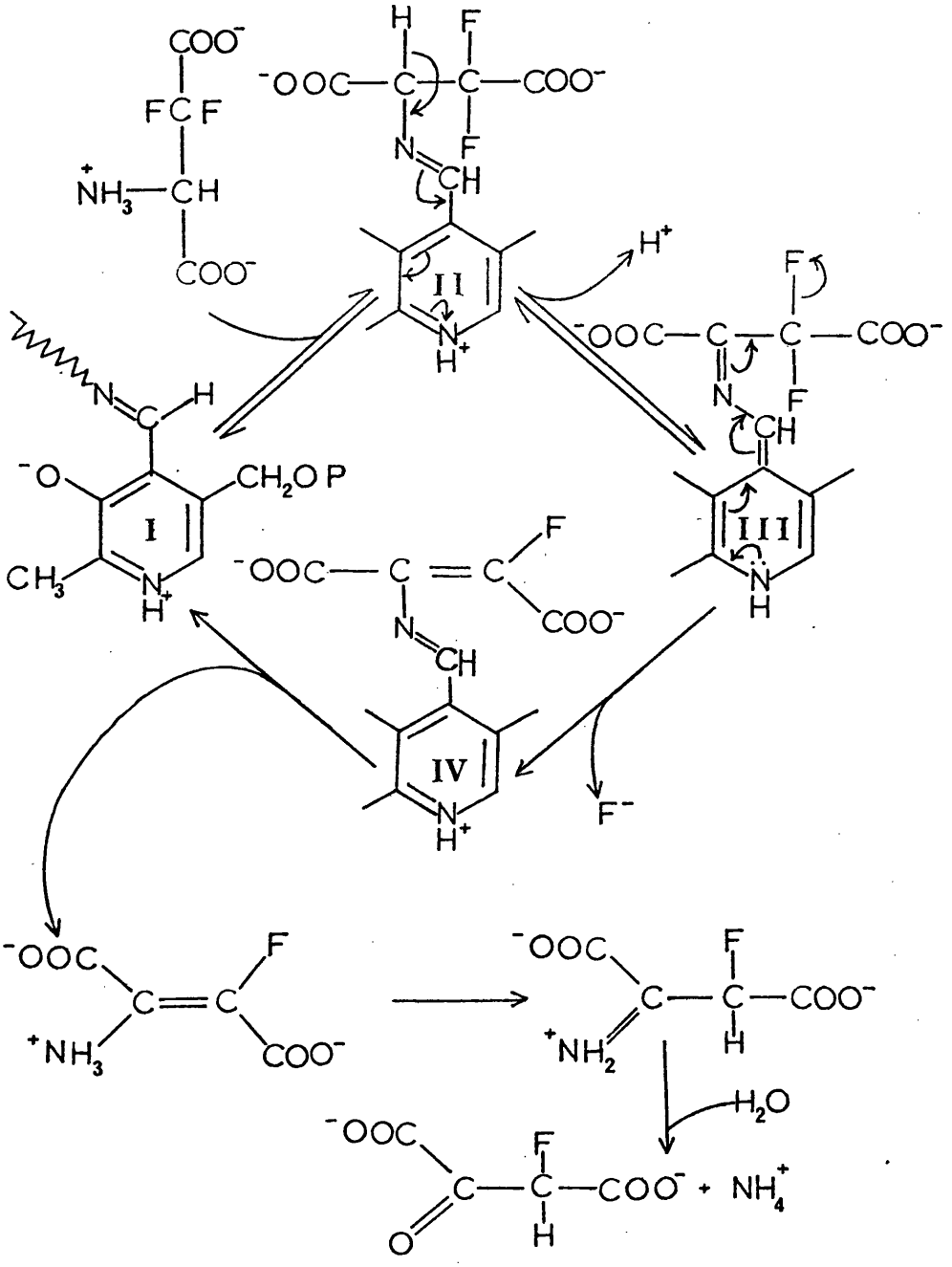
$k = ([S] / Km + [S]) k_{cat}$ ie. k = 1.75 x $10^{-4}s^{-1}$ found from this relationship.

The first order kinetics of the reaction are in accord with the scheme of Figure 54 where the enzyme-difluoro-aspartate complex with λ_{max} 340nm (X_1) is transformed into the enzyme species X_2. X_2 might reasonably be concluded to represent the aminic form of AAT, or the aminic enzyme difluoro-oxaloacetate complex with λ_{max} 328nm if transamination of difluoro-aspartate was occurring (Briley et al., 1977c).

However results of an attempt to measure a depletion of aldimine enzyme species (Fig. 59ii) indicate that the aminic form of AAT and difluoro-oxaloacetate are not primary products. The progress curve obtained

Figure 60

Possible mechanism for the α,β-elimination of difluoro-aspartate by the aldimine form of aspartate aminotransferase.



I ⟵ II Transaldimination

II ⟵ III Deprotonation

III ⟵ IV β-Elimination

IV ⟵ I Transaldimination

in this assay could possibly be interpreted as the formation of a keto-acid product without the concomittant formation of aminic enzyme. In view of the known propensity of aldimine-AAT to catalyse the α,β-elimination of substrate analogues with strongly electronegative β-substituents, (Kun et al., 1960; Manning et al., 1968; John and Fasella, 1969; John and Tudball, 1972; Morino and Okamoto, 1972), a reaction of this type ought to be considered. A possible mechanism for the α, β-elimination of difluoro-aspartate is presented in Figure 60 and is based on the similar mechanisms suggested by the above authors. To be noted are the products mono fluoro-oxaloacetic acid which is a substrate of malate dehydrogenase (Kun, 1969) and the aldimine form of aspartate aminotransferase. In support of a possible α,β-elimination of difluoro-aspartate are the spectral changes on prolonged incubation of difluoro-aspartate and aldimine-AAT (Fig. 53) in that the only absorption changes that might be expected would be minor changes in the ultraviolet due to accumulated enol form of a keto acid product.

However such a proposal would be in contradiction with the observed first order kinetics of the reaction with respect to enzyme concentration, unless, for example, the enzyme species X_2 represented a very tightly-bound aldimine enzyme-keto-acid complex (which is improbable on spectral grounds), or the product aldimine enzyme was inactive in transforming more difluoro-aspartate

In connection with this latter proposal it has been observed that concomittant with α,β-elimination of β-chloro-L-alanine (Morino and Okamoto, 1972) and L-serine-O-sulphate (John and Fasella, 1969), enzyme inactivation also occurs. This inactivation has been shown to be due to the covalent modification of the active site lysine residue by the bound substrate molecule in both cases and additionally of the cysteine 390 residue in the case of L-serine-O-sulphate (John et al., 1973; Morino et al., 1974). However in the present study inactivation of AAT (at least with respect to the normal catalytic cycle) was not observed (Fig. 59ii).

The reaction of L-serine-O-sulphate with aldimine-AAT (John and Fasella, 1969) is of particular interest since it also involves transamination. Concurrent transamination and α,β-elimination of difluoro-aspartate is not contra-indicated by the results of the attempt to demonstrate a depletion of aldimine enzyme species. This arises because any mono fluoro-oxaloacetate acid produced by β-elimination would react rapidly with aminic enzyme produced by transamination to give aldimine enzyme, oxaloacetate, F^- and NH_4^+ (Kun et al., 1960).

Thus, in conclusion, it is evident that the preliminary study of the substrate activity of difluoro-aspartate is insufficient to characterise the reaction.

Evidence for the nature of X_2 or indeed direct proof of the production of difluoro-oxaloacetate remain lacking, although the known specificity of malate dehydrogenase limits the possible alternative products.

Molecular nature of the aldimine AAT-difluoro-aspartate complex with λ_{max} 340nm.

Some indications of the molecular nature of the complex with λ_{max} 340nm can be obtained from the circular dichroism (CD) studies.

AAT exhibits an anomalous optical rotatory dispersion (ORD) curve with a positive Cotton effect (in the terminology of Djerassi, 1960) in the absorption bands of the PALP chromophore. This induced optical activity, ('extrinsic' Cotton effect) is a result of the tight binding of the symmetrical PALP within an assymmetrical environment. The phenomenon has been observed in the absorption bands of other protein-bound coenzymes (Ulmer and Vallee, 1965) and has been used as a technique with which to study enzyme-sustrate interactions.

The 'extrinisic' Cotton effect in the absorption bands of AAT and its complexes with specific ligands have been studied in detail by means of spectropolarimetry and with higher sensitivity by the recording of circular dichroism (CD) spectra (Torchinsky and Koreneva, 1963; 1964a; 1964b; Torchinsky et al., 1968; Fasella and Hammes, 1964; 1965; Breusov et al., 1964; Martinez-Carrion et al., 1970a; Harris and Bayley, 1975). These studies have underlined the profit of such measurements. Moreover the work of Torchinsky and his associates has

demonstrated the value of detecting differences in Cotton effects as a method for distinguishing different forms of the enzyme with otherwise identical absorption spectra.

Torchinsky and Koreneva (1963, 1964a, 1964b) have shown that carbonyl reagents (eg. hydroxylamine; hydrazine) that cause the rupture of the 'internal' PALP-lysine aldimine lead to the loss of the positive Cotton effect also. Carbonyl reagents with bulky substituents (eg. isonicotine hydrazide, semicarbazide) however, cause its replacement by a negative Cotton effect, whereas the binding of dicarboxylic acids merely shifted the Cotton effect, in conformity with changes in the absorption spectrum.

These results suggest that the observed Cotton effect of AAT is due to the presence of the aldimine linkage. Such an effect could be explained by an out of plane constraint placed on the conjugated C_4' - N bond of the internal aldimine by the apo-protein and permitted by rotation about C_4-C_4'. Rupture of this bond by hydroxylamine would then understandably abolish optical activity.

Although these conclusions are subject to debate (see e.g. Torchinsky et al., 1968; Ivanov and Karpeisky, 1969) the work of Torchinsky and Koreneva (1963, 1964a, 1964b) with carbonyl reagents suggests an empirical rule; rupture of the internal aldimine linkage results

in loss of optical activity. This rule was extended to the reaction of AAT with the substrates and substrate analogues.

Most notable of the substrate analogues studied was α-methylaspartate, the addition of which to aldimine-AAT results in a spectrum with absorption maxima at 360nm and 430nm indistinguishable from that obtained upon addition of a dicarboxylic acid. However ORD measurements demonstrated that the absorption band at 360nm was only weakly optically active and that the band at 430nm was devoid of optical activity in complete contrast to the ORD curve for an aldimine-AAT-dicarboxylic acid complex. Accordingly it was suggested that the interaction of α-methylaspartate with aldimine-AAT results in transaldimination to the substrate-aldimine Schiff base. Subsequent chemical studies (Malakohava and Torchinsky, 1965) and kinetic studies (Hammes and Haslam, 1968) have established the validity of this proposal for the α-methlyaspartate complex.

In the present work, titration of aldimine-AAT with difluoro-aspartate at either pH 7.4 or pH 5.5 resulted in the loss of the predominant circular dichroism band (365nm, pH 7.4 Fig. 47; 431nm, pH 5.5 Fig. 50) and the formation of a new weak dichroic maximum at 345nm, in both cases corresponding to the new absorption maximum at 340nm. Moreover values found for the dissociation constant at pH 7.4 (K = 2.1mM) and pH 5.5 (K = 11.0mM)

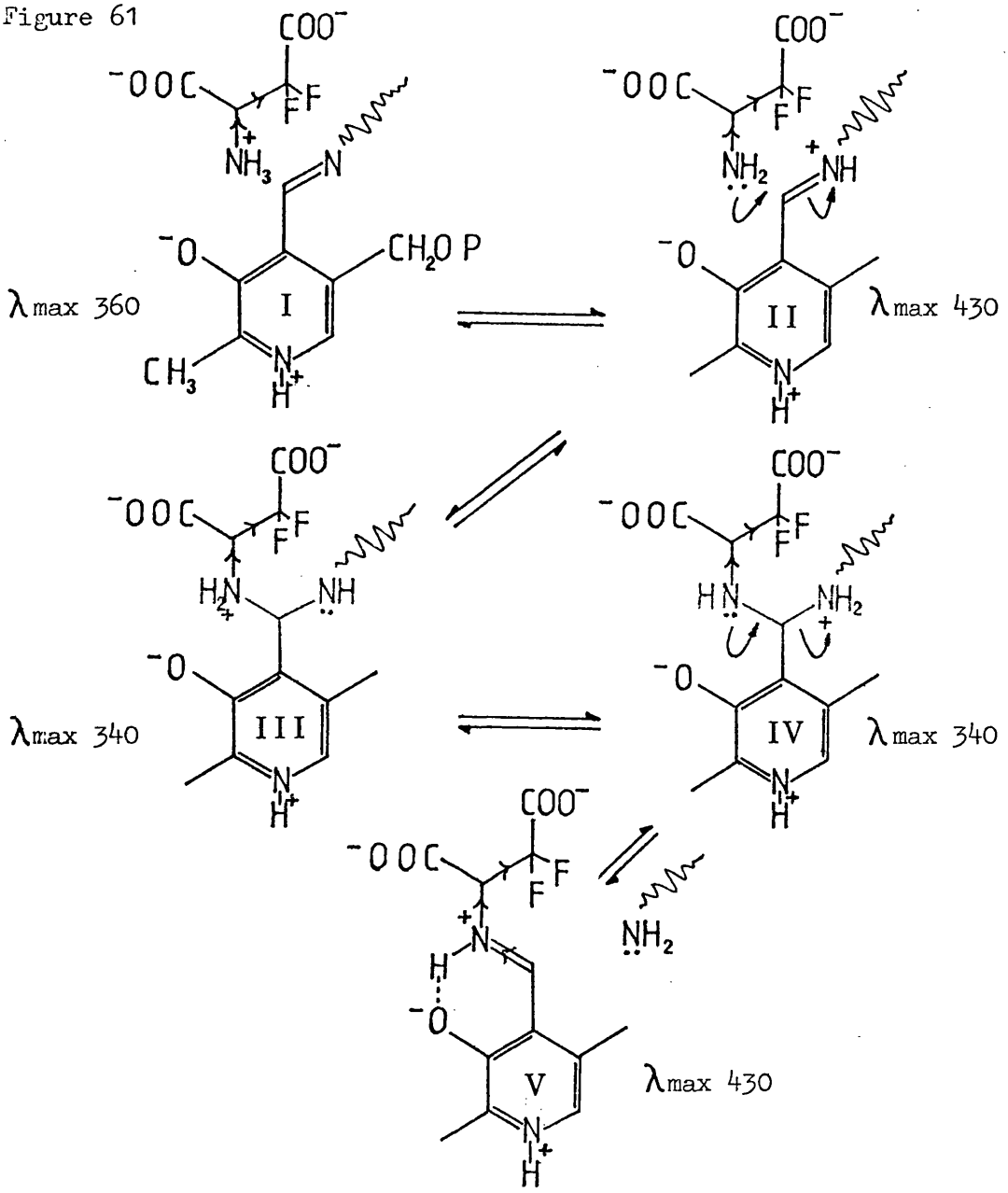
are in excellent agreement with the values found by spectrophotometric titration, indicating that the observed changes of circular dichroism are a result of the formation of the aldimine-AAT-difluoro-aspartate complex with λ_{max} 340nm.

The dissymmetry factors (anisotropy ratios) exhibited by the AAT-difluoro-aspartate complex are approximately 45% (at pH 7.4) and 30% (at pH 5.5) of that exhibited by the unbound enzyme (Table 5). This loss of optical activity is comparable to that observed on formation of the α-methylaspartate complex at 360nm (Martinez-Carrion et al., 1970a).

Considering the work of Torchinskii and Koreneva (1964) the covalent congress of difluoro-aspartate and enzyme-bound PALP seems highly likely. These conclusions are further supported by the effect of formate on the spectrum of aldimine-AAT, where covalent interaction is improbable. Addition of formate (3.5M) to aldimine-AAT causes a shift in the absorption maximum from ca 362nm to ca 345nm with the concomittant rise of a minor absorption band at ca 430nm. Yet in contrast to the interaction of difluoro-aspartate these absorption bands exhibit a similar anistropy ratio to that of unbound enzyme (Morino et al., 1974).

Of especial interest is the optical activity of the 430nm minor absorption band of the aldimine-AAT-difluoro-

Figure 61



Proposed reaction of difluoro-aspartate with Aspartate Aminotransferase.

I Michaelis complex

I ⟵ II Proton transfer from the amino group of the substrate to the internal aldimine nitrogen to give the Michaelis complex II.

II ⟵ III Formation of the tetrahedral intermediate III.

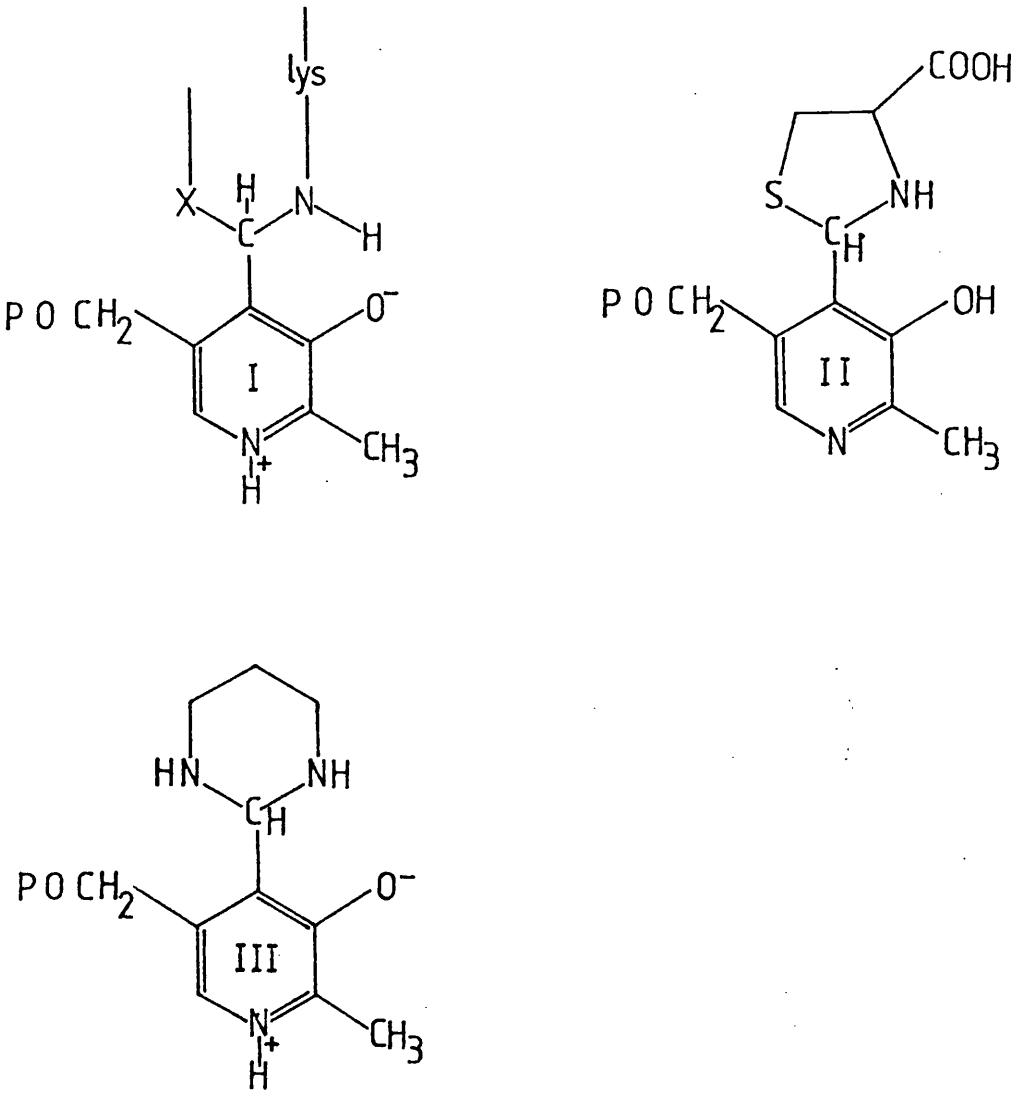
III ⟵ IV Proton transfer from the substrate amino group to the ε-amino group of lysine.

IV ⟵ V Completion of the transaldimination step, elimination of the ε-amino group of lysine to form the substrate aldimine V.

aspartate complex (Table 5). This absorption band at pH 5.5 (Fig. 39) is practically adichroic (Table 5) and direct analogy with the work of Torchinsky and Koreneva (1964a) would suggest that this absorption band was due to the PALP-difluoro-aspartate Schiff base. At pH 7.4 however the 430nm absorption band of the AAT-difluoro-aspartate complex has an anisotropy ratio 70% of that of the free enzyme at pH 7.4 which might seem to indicate that this absorption band is predominately due to the internal aldimine-Schiff base ie. a non covalent AAT-difluoro-aspartate complex.

The CD data discussed above indicates that the species having λ_{max} 340nm results from cleavage of the PALP-lysyl AAT Schiff base and formation of a covalent AAT-difluoro-aspartate complex. By analogy with the accepted mechanism of interaction of aldimine AAT with amino acids, the initially-most obvious structure for this complex is that of a Schiff base formed between aldimine AAT and difluoro-aspartate. The value of λ_{max} itself, however, argues against this, for it is generally true that species containing a C_4' = N double bond in conjugation with the pyridine ring have λ_{max} values of 360nm or greater. An alternative possibility is a tetrahedral intermediate (Fig. 61) arising from initial attack of difluoro-aspartate on the PALP-lysyl aldimine Schiff base. This species would be (cf. Ivanov and Karpeisky, 1969; Snell and DiMari, 1970; Severin and Gulaev, 1976; Metzler et al., 1978,

Figure 62



Substituted aldamine derivatives of Pyridoxal-5'-Phosphate.

I. Proposed structure for the 340nm chromophore of γ-subform-AAT.

II. Thiazolidine derivative formed between PALP and Cysteine.

III. Hexahydropyrimidine derivative formed between 1,3-diaminopropane and PALP.

Metzler, 1979) a necessary intermediate on route to an aldimine-difluoro-aspartate Schiff base and would be expected to have a λ_{max} value of approximately 340nm. Thus the γ-subform of AAT has λ_{max} 340nm which is unaffected by mild resolution (Scardi et al., 1963), glutamate, 2-oxoglutarate, $NaBH_4$, or by pH changes between 5 and 8.5 (Martinez-Carrion et al.,1965), and has been assigned (Martinez-Carrion et al., 1970) the structure shown in Figure 62 (structure I), where X is an unspecified nucleophilic group on the protein. This structure can be seen to be directly analogous to that suggested for the difluoro-aspartate-containing complex (Fig. 61). Furthermore a number of cyclic addition complexes of PALP with bidentate compounds see Figure 62 structure II, (Heyl et al., 1948; Beull and Hansen, 1960) and Figure 62, structure III (O'Leary, 1971) have similar structures to that proposed in Figure 61 and all have λ_{max} in the region of 330nm

Interaction of aldimine AAT with difluoro-aspartate has been shown (Section 7ii) to lead to the slow production of a keto-acid substrate for malate dehydrogenase. The K_m for this reaction corresponds to the dissociation constant for the aldimine AAT difluoro-aspartate species with λ_{max} 340nm (Section 4ii) suggesting that the latter is an intermediate in the pathway of keto-acid production. As discussed previously (page 239) it is likely that the pathway involves transamination (Banks et al., 1968b) or α,β-elimination (Antonini

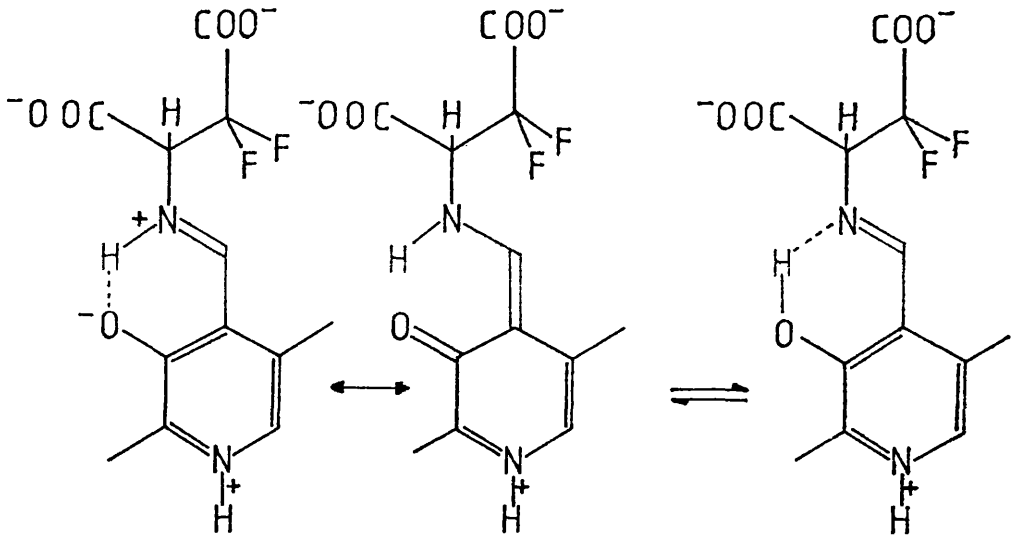
et al., 1970; John and Tudball, 1972) mechanisms either of which normally involves a rate-determining loss of the C_α proton of the amino-acid substrate. The rates reported for this process (eg.k = $250s^{-1}$ for transamination of aspartate, Banks et al., 1968b; k_{cat} = $3s^{-1}$ for α, β -elimination of threo-β-chloroglutamate, Manning et al., 1968; and k_{cat} = $1.6s^{-1}$ for α, β -elimination of β-chloro-L-alanine, John and Tudball, 1972) are much smaller than the lower bound apparent pseudo first order rate constant (k = $924s^{-1}$), calculated on the basis of stopped-flow data for the formation of the aldimine AAT-difluoro-aspartate complex with λ_{max} 340nm. This indicates that the latter species occurs before loss of the $C\alpha$ proton, and ketimine intermediates, known to have λ_{max} in the 330nm-340nm region, (Fasella et al., 1966; Hammes and Haslam, 1969) can be discounted as candidates for the difluoro-aspartate complex with λ_{max} 340nm.

No spectrally-distinct intermediate with λ_{max} 340nm, corresponding to that proposed here, has been reported in the reactions of AAT with natural amino-acid substrates. Such a tetrahedral species is, as mentioned above, an accepted precusor of the aldimine Schiff base in transamination reactions, and its stabilization relative to the Schiff base in the case of difluoro-aspartate can readily be rationalized in terms of the inductive effects of fluorine. Thus, conversion of the tetrahedral complex into the Schiff base (Fig. 61)

involves participation of the difluoro-aspartyl nitrogen lone-pair in displacing the lysyl-NH_2^+ grouping, an event made less likely by the negative inductive effects of the CF_2 grouping (Chambers, 1973). Conversely, reversion of the Schiff base to the tetrahedral intermediate would be facilitated by the same factor (c.f. Middleton and Krespan, 1965).

Circular dichroism evidence (page247) suggested, at least at pH 5.5, that the minor spectral peak with λ_{max} 430nm might correspond to the Schiff base shown in Figure 61. The presence of relatively low concentration of this Schiff base in an equilibrium system involving the tetrahedral species and a PALP-lysyl aldimine Schiff base is consistent with the results of the borohydride reduction experiments. These experiments (Section 5) were designed to trap a Schiff base of the type shown in Figure 61. Addition of sodium borohydride to a mixture of AAT and difluoro-aspartate led to an immediate loss of the 340nm absorption maximum and to the appearance of a new maximum at 330nm. The use of radiolabelled borohydride established that only ca 4% of the enzyme-bound radioactivity could subsequently be resolved from the protein under conditions designed to release a reduced PALP-amino acid complex. This suggests that the spectral change associated with borohydride addition reflects preferential reduction of a PALP-lysyl aldimine Schiff base and corresponding shift of equilibrium back toward the latter Schiff base

Figure 63



Possible Tautomerization in a pyridoxal-5'-phosphate-difluoro-aspartate Schiff base.

and away from the intermediate with λ_{max} 340nm. The small fraction of resolvable radioactivity could represent reduced PALP-difluoro-aspartate Schiff base although the amounts available precluded effective analysis. Previous workers (Riva et al., 1964; Malakhova and Torchinskii, 1965) have used similar protocols in different systems to isolate and identify reduced PALP-substrate Schiff bases but failed to quote yields of resolved complexes. Moreover Relimpo et al., (1975) have stated that the internal PALP-lysyl aldimine is reduced in preference to PALP-substrate aldimines. It may well be that initially-low concentrations of the latter species in the equilibrium make trapping by borohydride particularly unfavourable in the difluoro-aspartate-AAT system.

An equilibrium of the type described involving relatively high concentrations of the tetrahedral intermediate will serve to explain my available data, although alternative explanations cannot be totally excluded. It is, for instance, possible that the species with λ_{max} 340nm could be an enol-imine (Fig. 63) structure in which the imine nitrogen atom has lost its proton to the ring phenolic oxygen. Although there is no evidence for such an assignment involving AAT, Matsushima and Martell (1967b) have attributed a 340nm absorption maximum to a PALP-valine Schiff base in a model system.

The rate of production of 'Keto-àcid product' from aldimine AAT and difluoro-aspartate.

As discussed previously, it is most likely that the apparent production of keto-acid following the interaction of the aldimine form of AAT and difluoro-aspartate proceeds via abstraction of the $C\alpha$-proton of difluoro-aspartate, which is generally found to be rate-determining step in transamination and α,β-elimination reactions. Although the turnover of difluoro-aspartate is two orders of magnitude faster than equivalent reactions in model non-enzymic systems it is nevertheless six orders of magnitude slower than the AAT-catalysed transamination of aspartate; clearly some explanation must be sought for this difference.

According to the hypothesis of Dunathan (1966), transamination requires alignment of the conjugated π-bond system, including the pyridine ring and the C-4'-N-C grouping, in such a way that C-H bonds at C_α or C-4' can be orientated perpendicular to the conjugated system. The reaction of difluoro-oxaloacetate with the aminic form of AAT was found to proceed with a rate constant corresponding to removal of a proton at C-4' of $k = 1.25 \times 10^{-3} s^{-1}$ (Briley et al., 1977c) and the relative slowness of this reaction was attributed to the steric hinderance involved in achieving the conformation which, according to Dunathan (1966), is required for transamination to take place. This steric

hinderance was caused by the bulky fluorine atoms and was not present in the case of the natural substrate oxaloacetate. The rate of the slow reaction associated with release of α-keto acid from difluoro-aspartate in the presence of AAT is even slower, having k_{cat} = $2.68 \times 10^{-4} s^{-1}$, and it is likely that similar factors contribute to this low value. The same conformational requirement applies to relative orientation of the π-bond system and the C_α-proton prior to cleavage of the latter, whether the subsequent reaction involves transamination or β-elimination. Molecular models show very-considerable steric resistance to the attainment of the conformation required by Dunathan (1966), consistent with the scheme of Ivanov and Karpeisky (1969), in the case of an aldimine Schiff base between PALP and difluoro-aspartate. This steric resistance is directly attributable to the presence of bulky fluorine atoms and is not present in the case of the natural substrate aspartate. The importance of the correct orientation of the C_α-proton has been endorsed by the work of Weintraub *et al.*, (1976) who showed that the predicted populations of different pyridoxal-amino-acid Schiff bases present in the 'C_α-H perpendicular' conformation agreed in general with their rates of proton exchange. The pyridoxal-valine Schiff base with a predicted zero population of the required conformer did not exchange its α-proton.

In the case of difluoro-aspartate-AAT system, steric consideration of the type described above, together

with the presence of only relatively low concentrations of the PALP-difluoro-aspartate Schiff base, can reasonably be assumed to account for the observed slow production of keto-acid product.

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